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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/55, 9/22, 15/12, C07K 14/47, C12N 5/10, C12Q 1/68, 1/34, A61K 48/00 — A1

(11) International Publication Number: WO 99/10501

(43) International Publication Date: 4 March 1999 (04.03.99)

(21) International Application Number: PCT/US98/17214

(22) International Filing Date: 19 August 1998 (19.08.98)

(30) Priority Data:

60/056,904	22 August 1997 (22.08.97)	US
60/056,907	22 August 1997 (22.08.97)	US
60/072,192	6 January 1998 (06.01.98)	US

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: APOPTOSIS–ASSOCIATED NUCLEASE CPAN

(57) Abstract

A nuclease which is activated during apoptosis has been identified and its coding sequence isolated and decoded. Its potential as a target of therapeutics is realized by using it as a screen for developing drugs for activation or inhibition of the apoptotic pathway.

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APOPTOSIS-ASSOCIATED NUCLEASE CPAN

TECHNICAL FIELD OF THE INVENTION

This invention is related to proteins involved in programmed cell death.

BACKGROUND OF THE INVENTION

Apoptosis is a defined program by which cells commit suicide in response to specific environmental stimuli (Kerr et al., 1972). Once initiated, the biochemical cascade of cell execution results in distinctive morphological changes such as reduction in cytoplasmic volume, membrane blebbing, and the condensation and fragmentation of nuclear chromatin (Wyllie, 1980). Apoptosis is important for normal embryonic development (Ellis et al., 1991, Steller, 1995), tissue remodeling (Hinchliffe, 1981), the deletion of T cells in thymic selection (Smith et al., 1989), and in cell mediated cytotoxicity in response to pathogenic infections or other irreparable cell damage (Duke, 1991).

Induction of apoptosis through cell death receptors such as Fas and TNF RI results in the activation of the caspase family of cysteine proteases with specificity for aspartic residues (Tewari and Dixit, 1995, Alnemri et al., 1996). Caspases are cytosolic zymogens that become activated in a sequencial cascade of proteolytic cleavages (Nicholson et al., 1995, Enari et al., 1996). More than ten different caspases have been identified in mammalian cells. Homolgues to the C. elgans enzyme CED-3 (Yuan et al., 1993) have been identified in many species and include the DCP-1 enzyme of Drosophila (Song et al., 1997) and the mammalian enzyme caspase-3, also called CPP32/Yama/Apopain (Fernandez-Alnemri et al.,1994, Nichoson et al., 1995, Tewari and Dixit, 1995). Specific inhibitors of caspases can block apoptosis in Fas- and TNF RI-stimulated cells suggesting that caspases are essential for transducing death signals (Tewari and Dixit, 1995, Dubrez, 1996). Although the specific substrates of each of the caspases have not been identifed, activated caspase-3 has been shown to cleave poly ADP-ribose polymerase (Nicholson et al., 1995), Pak2 (Rudel and BoKoch, 1997, Lee et al., 1997), gelsolin (Kothakota et al., 1997), U1-associated 70KD protein (Ciaciola-Rosen et al., 1996), D4-GDI (Na et al., 1996), sterol-regulatory element-binding proteins (SREBPS)(Wang et al, 1995, 1996), huntingtin (Goldberg et al., 1996), DNA-dependent protein kinase (Ciaciola-Rosen, 1996, Song et al., 1996), and the nuclear lamins. Although most cleavages result in the inactivation of the targeted protein, several proteins such as Pak2 (Rudel and Bokoch, 1997, Lee et al., 1997) and gelsolin (Kothakota et

al., 1997)) have been shown to be activated following proteolytic removal of a key regulatory domain and may play a role in mediating some of the cytosolic and morphologic changes of apoptosis.

Mechanisms proposed to explain nuclease activation in apoptosis include alterations in chromatin structure (Alnemri and Litwack, 1990), fluxuations in calcium levels, and the proteolytic activation of a specific nuclease:inhibitor complex (Fraser et al., 1996, Kayalar et al., 1996). Until now, it has remained unclear how the apoptotic program triggers nuclease activation. Although there are several candidate nucleases that might play a role in apoptosis, none have been found to be activated by caspases.

Apoptosis plays a central role in many human diseases and offers many potential targets for therapeutic intervention. Aberrant control of apoptosis may underlie autoimmune diseases, immune deficiency, ischaemic cardiovascular and neurological injury, Alzheimer's disease, and cancer. In heart attacks and stroke, inhibition of cellular apoptosis may prevent the damage or destruction of tissue that often occurs following the initial traumatic event. In cancer, it is believed that tumor cells have blocked or disabled the normal apoptotic processes that control cell growth. The selective activation of apoptosis in tumor cells may be one mechanism to control cancer.

Thus there is a need in the art for identifying key components in the apoptosis pathway, as well as for identifying means for manipulating the initiation or termination of the pathway.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide an isolated subgenomic DNA molecule which encodes an active nuclease protein (CPAN).

It is another object of the present invention to provide a nucleic acid construct comprising a promoter which is operably linked to a segment of nucleic acid which encodes an active CPAN protein.

It is an object of the present invention to provide an isolated subgenomic DNA molecule which encodes an immunogenic polypeptide.

Another object of the invention is to provide a polynucleotide probe for CPAN.

Another object of the invention is to provide a method of digesting DNA.

Another object of the invention is to provide a method of purifying an inactive CPAN.

Another object of the invention is to provide a method of purifying an active CPAN protein.

It is still another object of the invention to provide methods of identifying compounds which inhibit apoptosis.

It is yet another object of the invention to provide methods of identifying compounds which activate apoptosis.

It is another object of the invention to provide a method of producing a protein which activates apoptosis.

These and other objects of the invention are achieved by providing one or more of the embodiments described below. In one embodiment, an isolated subgenomic DNA molecule is provided which encodes an active CPAN protein of approximately 40 kd as measured on SDS-polyacrylamide gel electropheretograms. The CPAN protein has an amino acid sequence as shown in SEQ ID NO: 2. Biologically active variants of CPAN are also provided.

According to another embodiment of the invention a nucleic acid construct is provided which comprises a promoter which is operably linked to segment of nucleic acid which encodes a 40 kd active CPAN protein as measured on SDS-polyacrylamide gel electropheretograms and has an amino acid sequence as shown in SEQ ID NO: 2. Biologically active variants of CPAN may also be used.

In yet another embodiment of the invention an isolated subgenomic DNA molecule is provided which encodes an immunogenic polypeptide of at least 6 contiguous amino acids of an active CPAN protein of approximately 40 kd as measured on SDS-polyacrylamide gel electropheretograms,. The CPAN protein has an amino acid sequence as shown in SEQ ID NO: 2.

According to still another embodiment of the invention a polynucleotide probe is provided. The probe comprises at least 12 contiguous nucleotides selected from the sequence shown in SEQ ID NO: 1.

According to yet another embodiment of the invention a method of digesting DNA is provided. The method comprises the step of:

contacting a preparation of nuclei-free DNA with an active CPAN nuclease which is approximately 40 kd protein as measured by SDS-polyacrylamide gel electrophoresis, whereby the DNA is digested to form nucleotides and/or random DNA fragments.

In another embodiment of the invention a method of purifying an inactive CPAN is provided. The CPAN has an apparent molecular weight of 130 kD as measured by size exclusion chromatography and of approximately 40 kd as measured by SDS-polyacrylamide electrophoresis. The method comprises the step of:

contacting a cytoplasmic preparation with poly-uridylic acid and collecting a fraction of proteins which do not bind to poly-uridylic acid .

According to still another aspect of the invention a method is provided for purifying an active CPAN protein having an apparent molecular weight of 40 kd as measured on SDS-polyacrylamide gel electrophoretograms. The method comprises the steps of:

contacting a mixture comprising an active CPAN with polyuridylic acid, whereby the active CPAN binds to the polyuridylic acid to form a bound complex;

separating the bound complex of CPAN and polyuridylic acid from other components of the mixture.

According to still another aspect of the invention a method is provided for identifying compounds which inhibit apoptosis. The method comprises:

contacting a test compound with an active CPAN protein having a molecular weight of approximately 40 kd as measured on SDS-polyacrylamide gel electropheretograms;

testing the CPAN for the ability to fragment DNA, wherein a test compound which inhibits CPAN fragmentation of DNA is a candidate compound for inhibiting apoptosis.

Another embodiment of the invention provides a method of identifying compounds which inhibit apoptosis. The method comprises:

contacting a test compound with a cell comprising a recombinant DNA construct encoding an active CPAN protein having a molecular weight of approximately 40 kd as measured on SDS-polyacrylamide gel electropheretograms;

testing the CPAN for the ability to fragment DNA, wherein a test compound which inhibits CPAN fragmentation of DNA is a candidate compound for inhibiting apoptosis.

In yet another embodiment of the invention a method of identifying compounds which activate apoptosis is provided. The method comprises:

contacting a test compound with an inactive caspase activated nuclease (CPAN) protein having a molecular weight of approximately 130 kD as measured by molecular sizing chromatography;

testing the CPAN for the ability to fragment DNA, wherein a test compound which activates CPAN to fragment DNA is a candidate compound for activating apoptosis.

According to a further aspect of the invention a method is provided for identifying compounds which activate apoptosis. The method comprises:

contacting a test compound with an inactive CPAN protein, wherein the inactive CPAN has a molecular weight of approximately 130 kD as measured by molecular sizing chromatography;

assaying for an apparent increase in molecular size of CPAN as measured by molecular size exclusion chromatography, wherein a test compound which causes CPAN to behave as if it has a large molecular size on molecular size exclusion chromatography is a candidate compound for activating apoptosis.

A further aspect of the invention provides another method of identifying compounds which activate apoptosis. The method comprises:

contacting a test compound with a cell comprising an inactive CPAN protein having a molecular weight of approximately 130 kD as measured by molecular sizing chromatography;

testing the CPAN for the ability to fragment DNA, wherein a test compound which activates CPAN to fragment DNA is a candidate compound for activating apoptosis.

Still another aspect of the invention is another method of identifying compounds which activate apoptosis. The method comprises:

contacting a test compound with a cell comprising an inactive CPAN protein having a molecular weight of approximately 130 kD as measured by molecular sizing chromatography;

assaying for an apparent increase in the molecular size on molecular size exclusion chromatography of the CPAN protein in the cell, wherein a test compound which causes CPAN to behave as if it has increased in molecular size is a candidate compound for activating apoptosis.

According to another aspect of the invention a method of making a CPAN protein is provided. The method comprises the steps of:

culturing a recombinant cell in a suitable culture medium, wherein the recombinant cell comprises at least one polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1 and at least one polynucleotide comprising the nucleotide sequence of SEQ ID NO: 3, whereby a 40 kD CPAN protein and a 45 kD DFF45protein are expressed; and

recovering CPAN protein from the cultured recombinant cell.

The present invention thus provides the art with an attractive target for manipulating apoptosis. Activators and inhibitors can be identified which can be used therapeutically. By activating the nuclease apoptosis can be initiated, and by inhibiting the nuclease apoptosis can be blocked. Thus the art is provided with an important handle into the control of cell death.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Appearance of apoptotic activity in Fas-stimulated cell extracts. Jurkat cells were induced with the anti-Fas Ab and harvested at the indicated time points. Cytosolic extracts were prepared and assayed for caspase activity as measured using DEVD-AFC (Fig. 1A, solid circles), and for in vitro apoptosis activity in added naive nuclei as visualized by either percentage of nuclei exhibiting apoptotic morphology (Fig. 1A, open triangles) or by DNA fragmentation (Fig. 1C). Total cellular DNA was also extracted from apoptotic Jurkat cells at the indicated time points and analyzed for DNA fragmentation (Fig. 1B).

Figure 2. Caspase-3 activation of nuclease activity in cytosolic extracts. Caspase-3 was added to unactivated Jurkat cell cytosol and then tested in the in vitro apoptosis assay or for nuclease activity on naked DNA. Caspase-3 alone (lanes 1, 6) or cytosol alone (lanes 2, 7) are inactive in both assays.

Figure 3. Fig. 3A shows CPAN apoptotic activity as a function of caspase-3 concentration. EDTA inhibits the reaction. Fig. 3B shows nuclease activity as a function of CPAN concentration at constant caspase-3 concentration..

Figure 4. Analysis of Poly-U column fractions in the in vitro apoptosis assay (Fig. 4A) showed that the apoptotic activity eluted late in the gradient after most of the contaminating proteins. The peak correlated with the elution of a peak of nuclease activity (Fig. 4B). SDS-PAGE analysis (Fig. 4C) of the column fractions revealed a single 40 KD band which correlated with both nuclease and apoptotic activity.

Figure 5. The Poly-U purified, activated form of CPAN was resolved on a Sephacryl S-200 column, and fractions were assayed for in vitro apoptosis (Fig. 5A) and nuclease activity (Fig. 5C). A single peak of both apoptotic and nuclease activity was detected eluting in the void volume of the column. Fig. 5B shows SDS-PAGE of the column fractions. A 40 KD band correlated with both nuclease and apoptotic activity.

Figure 6. Amino acid residues of CPAN were identified. Degenerate oligonucleotides encoding the amino- and carboxy-terminal ends of peptide IV (underlined) were used to amplify a 70 bp DNA fragment of the predicted size from a human placenta

library. The unique DNA sequence obtained from the center of the PCR product between the two degenerate probes was used to do nested PCR which identified a 400 bp fragment encoding peptides IV, V, and VI, as shown.

Figure 7. Northern blot analysis with the CPAN cDNA of human tissues is shown in Fig. 7A. A 3.5 kb mRNA band was detected in all tissues examined and was most abundant in pancreas. A large molecular weight band (>10 kb) was also detected in several tissues. Northern blot analysis of eight human cancer cell lines (Fig. 7B) indicated that CPAN is more abundant in promyelocytic leukemia (HL-60), a colorectal carcinoma (SW480), and a melanoma (G361), but was almost undetectable in Burkitt's lymphoma (Raji) and lung carcinoma (A549).

Figure 8. CPAN alone (Fig. 8A, lane 1) was expressed poorly in cells and was recovered only when cells were extracted with SDS-lysis buffer. Extraction with the detergent Triton X-100 resulted in no detectable CPAN being recovered (lane 2). When CPAN was expressed in the presence of DFF45 however (lane 4), CPAN was expressed at higher levels and was recovered in mild detergent buffer. Expression of DFF45 alone (Fig. 8B, lanes 1 and 3) resulted in a high yield of DFF45 in the presence or absence of SDS.

CPAN from the transfections was immunoprecipitated with an anti-HA antibody and then assayed for nuclease activity with and without caspase-3 treatment. As shown in Figure 8C, lanes 1-5, none of the samples were active prior to caspase-3 treatment. After caspase-3 treatment, however, the CPAN/DFF45 sample displayed nuclease activity and cleaved naked human DNA. To complete the analysis, we immunoprecipitated DFF45 from the same transfections using the anti-gluglu antibody and tested for nuclease activity as described above. As shown in Fig. 8C, none of the samples were active prior to caspase treatment and only the co-transfection containing CPAN and DFF45 displayed nuclease activity after treatment.

Fig. 8D shows Western blot analysis of CPAN and DFF45 before and after caspase treatment.

Figure 9. Caspase activity induced by staurosporine in cells transfected with CPAN and DFF45 is the same as that in cells transfected with DFF45 alone (Fig. 9A). Nonetheless, the nuclease activity induced in the two types of transfectants indicates that only cells transfected with CPAN have nuclease activity (Fig. 9B).

Figure 10. Dissociation of DFF45 fragments from activated CPAN. KT3-tagged CPAN was co-expressed with 6xHis tagged DFF45 in insect cells, purified on a KT3 MAb column, and eluted with KT3 peptide. The purified CPAN/DFF45 complex was treated with

caspase-3 and then purified by Poly-U Sepharose chromatography. SDS-PAGE analysis (Fig. 10A) of the KT3-purified CPAN/DFF45 complex (lane 1), the caspase-3 treated complex (lane 2), the Poly-U column flow-through (Poly-U FT, lane 3), the eluate from the Poly-U column (lane 4), and purified 6xHis-DFF45 expressed alone in insect cells (lane 5) is shown. The apoptotic activity of the purified CPAN samples was determined in the in vitro apoptosis assay (Fig 10B). Total genomic DNA was extracted, analyzed by 1% gel electrophoresis and DNA fragmentation visualized by ethidium bromide staining.

DETAILED DESCRIPTION OF THE INVENTION

It is a discovery of the present inventors that an inactive nuclease complex is directly activatable by the protease caspase-3; the activated nuclease is responsible for the nuclear degradation which occurs during apoptosis. The nuclease complex has been isolated in inactive form, cleaved *in vitro* using caspase-3, and used in *in vitro* DNA digestion reactions. The activated nuclease, which is dubbed CPAN for Caspase Activated Nuclease has a molecular weight on SDS-polyacrylamide gels of about 40 kD and an amino acid sequence as shown in SEQ ID NO: 2. Moreover, it has been found that active CPAN binds avidly to poly-uridylic acid, but the inactive form does not. In addition, the cDNA encoding CPAN has been isolated and its sequence determined.

Expression of CPAN has been achieved in recombinant cells. Such expression apparently requires the co-expression of the DFF45 protein. DFF45 is a protein of about 45 kD which binds to CPAN and negatively regulates its activity. Upon activation with caspase-3, DFF45 is cleaved into fragments which dissociate from CPAN, leaving an active nuclease. CPAN cannot be activated when the cleavage site at Asp 117 in DFF45 is mutated

The subgenomic DNA which encodes CPAN is a molecule of less than the whole human chromosome on which its resides. It can be genomic DNA or cDNA. Although one particular sequence has been found, variants of the sequence from other individuals, from other mammalian species, and even synthetically designed, are encompassed herein. Some variants will encode the same protein, but differ only by virtue of degeneracy of the genetic code. Other variants will differ by up to 2, 5, 20, or 15%, yet still be within the scope of the claims. Such variants can be isolated by hybridization to other individual's DNA, from other organs, from other mammals' DNA, etc. Each such variant can be tested to be sure that it encodes a protein that retains the nuclease activity.

Any naturally occurring variants of the CPAN sequence that may occur in human tissues and which has nuclease activity are within the scope of this invention. Thus, reference herein to either the nucleotide or amino acid sequence of CPAN includes reference to naturally occurring variants of these sequences. Nonnaturally occurring variants which differ by as much as four amino acids and retain biological function are also included here. Preferably the changes are conservative amino acid changes, *i.e.,* changes of similarly charged or uncharged amino acids.

As discussed above, minor amino acid variations from the natural amino acid sequence of CPAN are contemplated as being encompassed by the term CPAN; in particular, conservative amino acid replacements are contemplated. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are generally divided into four families: (1) acidic=aspartate, glutamate; (2) basic=lysing, arginine, histidine; (3) non-polar=alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar=glycine, asparagine, glutamine, cystine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the enzymatic properties of the resulting molecule, especially if the replacement does not involve an amino acid at a binding site involved in the interaction of CPAN or its derivatives with DNA. Whether an amino acid change results in a functional peptide can readily be determined by assaying the specific enzymatic properties of the CPAN polypeptide derivative. Assays are described in detail below.

Polynucleotide molecules encoding the nuclease can be linked to a promoter to put the expression of the nuclease under the control of the promoter. Linkage of two DNA sequences using DNA ligase is well known in the art. Any promoter having desirable characteristics can be selected and used. Such selection and use are well within the skill of the art. The constructs can be used in vectors for introduction into host cells. The host cells can be transfected with the DNA construct *in vitro* or *in vivo.* Host cells grown *in vitro* can be used to express the nuclease. Host cells transfected *in vivo* can be therapeutically treated for destruction. For example, if the cells are tumor cells, introduction of the CPAN gene could cause the cells to enter the apoptotic pathway. In order to control expression of CPAN, regulatable promoters can be used which are only active in the presence of a specific inducer

molecule. Alternatively, in *E. coli,* CPAN may be expressed in inclusion bodies, which would keep them inactive. Another possible route to expression of CPAN is in baculovirus infected cells which expire after infection, in any event. Still another possible expression route is by linking the protein coding sequence to a leader or signal sequence which will cause the protein to be secreted from the cells.

According to a particularly desirable configuration, the promoter which is used to control transcription of CPAN is only active in tumor cells. Thus the promoter is one which is activated or derepressed in tumor cells. Such promoters include but are not limited to - fetaprotein, carcinoembryonic antigen, and c-erbB2, 3, 4 promoters.

According to another therapeutic aspect of the invention antisense constructs containing the CPAN antisense strand operatively linked to a promoter, are administered to cells which are in danger of cell death after a trauma. For example, after a stroke or a heart attack, cells can be treated with an antisense CPAN construct to prevent undesirable cell death. Any disease state involving aberrant or undesirable apoptosis can be so treated.

Portions of the CPAN-coding polynucleotides can also be useful. These can be useful as probes or primers for detecting cells expressing CPAN. These can also be useful to express immunogenic portions of CPAN, such as epitopes which are particularly useful for raising antibodies against CPAN. Such polynucleotides preferably encode at least 6, 8, 10, or 20 contiguous amino acids of the CPAN protein, preferably 6 contiguous amino acids of the CPAN protein sequence shown in SEQ ID NO: 2. Probes and primers according to the present invention are at least 12, 14, 16, 18, 20, 25, or 30 contiguous nucleotides of the sequence of CPAN. These may also contain other sequences, such as restriction enzyme sites, promoters, translation initiations sites, etc. For expressing epitopes of CPAN slightly longer molecules may be desired. If the molecules encode at least 6, 8, 10, 12, or 14 contiguous amino acids of CPAN, then they are useful as immunogen expressers or as antigen expressers for use in immunological assays.

The polynucleotides and constructs comprising CPAN sequences can also be placed in vectors for various purposes. Vectors are useful for replication in the host cells, so that homologous recombination is not required for maintenance. Suitable vectors for various purposes are well known in the art and are within the skill of the artisan to select. Similarly host cells for use with various constructs and vectors are similarly well known and can be selected at will for their known properties.

Particular constructs are described below. However, as will be apparent to one skilled in the art,other constructs can be constructed and purified using standard recombinant

DNA techniques as described in, for example, Sambrook et al. (1989), MOLECULAR CLONING: A LABORATORY MANUAL, 2nd ed. (Cold Spring Harbor Press, Cold Spring Harbor, New York); and under current regulations described in United States Dept. of HHS, NATIONAL INSTITUTE OF HEALTH (NLH) GUIDELINES FOR RECOMBINANT DNA RESEARCH. The polypeptides of the invention can be expressed in any expression system, including, for example, bacterial, yeast, insect, amphibian and mammalian systems. Expression systems in bacteria include those described in Chang et al., Nature (1978) 275: 615, Goeddel et al., Nature (1979) 281: 544, Goeddel et al., Nucleic Acids Res. (1980) 8: 4057, EP 36,776, U.S. 4,551,433, deBoer et al., Proc. Natl. Acad. Sci. USA (1983) 80: 21-25, and Siebenlist et al., Cell (1980) 20: 269. Expression systems in yeast include those described in Hinnen et al., Proc. Natl. Acad. Sci. USA (1978) 75: 1929; Ito et al., J. Bacteriol. (1983) 153: 163; Kurtz et al., Mol. Cell. Biol. (1986) 6: 142; Kunze et al., J. Basic Microbiol. (1985) 25: 141; Gleeson et al., J. Gen. Microbiol. (1986) 132: 3459, Roggenkamp et al., Mol. Gen. Genet. (1986) 202 :302) Das et al., J. Bacteriol. (1984) 158: 1165; De Louvencourt et al., J. Bacteriol. (1983) 154: 737, Van den Berg et al., Bio/Technology (1990) 8: 135; Kunze et al., J. Basic Microbiol. (1985) 25: 141; Cregg et al., Mol. Cell. Biol. (1985) 5: 3376, U.S. 4,837,148, US 4,929,555; Beach and Nurse, Nature (1981) 300: 706; Davidow et al., Curr. Genet. (1985) 10: 380, Gaillardin et al., Curr. Genet. (1985) 10: 49, Ballance et al., Biochem. Biophys. Res. Commun. (1983) 112: 284-289; Tilburn et al., Gene (1983) 26: 205-221, Yelton et al., Proc. Natl. Acad. Sci. USA (1984) 81: 1470-1474, Kelly and Hynes, EMBO J. (1985) 4: 475479; EP 244,234, and WO 91/00357. Expression of heterologous genes in insects can be accomplished as described in U.S. 4,745,051, Friesen et al. (1986) "The Regulation of Baculovirus Gene Expression" in: THE MOLECULAR BIOLOGY OF BACULOVIRUSES (W. Doerfler, ed.), EP 127,839, EP 155,476, and Vlak et al., J. Gen. Virol. (1988) 69: 765-776, Miller et al., Ann. Rev. Microbiol. (1988) 42: 177, Carbonell et al., Gene (1988) 73: 409, Maeda et al., Nature (1985) 315: 592-594, Lebacq-Verheyden et al., Mol. Cell. Biol. (1988) 8: 3129; Smith et al., Proc. Natl. Acad. Sci. USA (1985) 82: 8404, Miyajima et al., Gene (1987) 58: 273; and Martin et al., DNA (1988) 7:99. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts are described in Luckow et al., Bio/Technology (1988) 6: 47-55, Miller et al., in GENETIC ENGINEERING (Setlow, J.K. et al. eds.), Vol. 8 (Plenum Publishing, 1986), pp. 277-279, and Maeda et al., Nature, (1985) 315: 592-594. Mammalian expression can be accomplished as described in Dijkema et al., EMBO J. (1985) 4: 761, Gorman et al., Proc. Natl. Acad. Sci. USA (1982b) 79: 6777,

Boshart et al., Cell (1985) 41: 521 and U.S. 4,399,216. Other features of mammalian expression can be facilitated as described in Ham and Wallace, Meth. Enz. (1979) 58: 44, Barnes and Sato, Anal. Biochem. (1980) 102: 255, U.S. 4,767,704, US 4,657,866, US 4,927,762, US 4,560,655, WO 90/103430, WO 87/00195, and U.S. RE 30,985.

CPAN can be used to directly digest DNA. This requires no intermediates upon which CPAN acts. CPAN directly digests DNA into random fragments and/or nucleotides. To perform the digestion *in vitro* however, it has been found that it is desirable to have at least 0.2 mg protein concentration. For example, using at least 0.2 mg/ml of a molecular carrier such as bovine serum albumin is beneficial. Preferably between 0.2 and 2 mg/ml is used. More preferably between 0.5 and 1.5 mg/ml is used. Other proteins can be used to fulfill this function, so long as they do not inhibit the nuclease activity. Whereas nuclei can be used as a substrate for the enzyme, isolated DNA may also be used to simplify the reaction mixture. Isolated DNA is readily available commercially, such as from salmon sperm.

Purification of CPAN, in its active form, is facilitated by the use of an affinity property of the active enzyme. The active enzyme binds to poly-uridylic acid. Thus affinity columns of poly-uridylic acid can be readily used to separate active from inactive CPAN, as well as purifying active CPAN from other proteins in the cytoplasm. Proteins which bind to the poly-uridylic acid can be eluted using an ionic salt, preferably in a concentration gradient to effect a higher degree of purity. It has also been found that S-Sepharose HP™ and molecular sizing columns can be used to enhance the degree of purity of the CPAN preparations. These have been used on preparations prior to loading on the poly-uridylic acid affinity columns. See Table 1, which provides information on a particular purification scheme which achieves greater than 11,000-fold purification.

Inhibitors of apoptosis are desirable for a number of therapeutic applications. For example, where cells are damaged and the apoptotic pathway is initiated, such as by a heart attack or stroke, it may be desirable to salvage the cells and provide them with time to recover. Assays for identifying inhibitors of CPAN may provide inhibitors of apoptosis which can be used therapeutically. Simple assays can be set up, in which test compounds are contacted with CPAN, and the ability of CPAN to fragment DNA is tested. Preferably this is done in a system in which isolated nucleic acids are provided as a substrate, although isolated nuclei can also be used. DNA fragmentation can be assayed by any means known in the art, including but not limited to electrophoresis on a gel, staining nuclei with TUNEL, and photometric enzyme immunoassay for the determination of cytoplasmic histone-associated

DNA fragments. Cells which are contacted with a test agent may contain recombinant constructs which express active CPAN.

Candidate agents for testing as inhibitors or activators can be small molecules, peptides, peptoids, nucleic acids, etc. The agents may have known functions or no previously known function. Whole libraries of compounds can be tested in groups of compounds or individually. If a particular type of compound shows inhibitory or activating activity, then analogues and variants of that type of compound can be tested for improved activity.

Another method for assaying for inhibition of CPAN is by assaying the molecular size of the protein by molecular size exclusion chromatography. The CPAN protein when activated behaves as a much larger protein, such as $>10^6$ molecular weight.

Methods for identifying compounds which activate apoptosis are generally the converse of those for identifying inhibitors. One starts with the inactive CPAN precursor, *i.e.*, the complex of CPAN and DFF45, or a cell which contains the inactive CPAN precursor. The protein or cell containing it is contacted with various test agents. Those which can cause the inactive protein to fragment DNA, or cause the protein to have an apparent molecular weight on molecular size exclusion chromatography of $>10^6$, are agents which can potentially activate apoptosis.

Antibodies are also provide herein, which are specifically reactive with CPAN protein. These can be made by immunizing animals with a preparation of the protein or polypeptide portions of the protein or fusion proteins comprising the portions of the protein. The antibodies can be polyclonal or monoclonal. Techniques for making such antibodies are well known in the art. Such antibodies may be used to inhibit CPAN protein activity and can be used to identify portions of CPAN protein to which small molecule inhibitors can be targeted.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

Apoptotic activity in Fas-stimulated cytosolic extracts

Induction of apoptosis in Jurkat cells by crosslinking of the Fas death receptor initiates a cascade of biochemical and morphological changes that ultimately lead to the death of the cell. To study this process in a cell-free system, we developed an in vitro apoptosis assay in which apoptotic cell extracts were added to naive nuclei and allowed to progress through the downstream events in the apoptotic cascade. Nuclei were analyzed for morphological changes following Hoechst staining and for DNA fragmentation after extraction of genomic DNA and analysis by agarose gel electrophoresis. Within one hour after treatment of Jurkat cells with the anti-Fas antibody, caspase activity was detected using the fluorometric substrate, DEVD-AFC (Fig. 1A). Caspase activity continued to increase until two hours and then decreased slowly over the remaining time course.

When cytosolic extracts were prepared and tested in the in vitro apoptosis assay, a peak of apoptotic activity was observed which correlated with the appearance of caspase activity (Fig. 1A). Apoptotic activity was calculated as the percentage of total nuclei that displayed the apoptotic morphology of chromatin condensation and margination (Fig. 1B). To confirm that the morphological changes observed reflected an apoptotic process, we extracted the DNA from the in vitro apoptosis assay samples and measured DNA fragmentation (Fig. 1C). The samples which displayed the most activity measured morphologically also showed the most significant DNA fragmentation, detected as an internucleosomal DNA ladder. The appearance of apoptotic activity in cytosol following the rise in caspase activity suggested that caspases may directly or indirectly activate a cytosolic factor that causes morphological changes in added nuclei and internucleosomal DNA fragmentation. The apparent decrease in apoptotic activity detected late in the time course may reflect the instability of the cytosolic activity or the depletion of this activity from the cytosolic compartment

General Methods and Materials

Caspase substrate DEVD-AFC and inhibitors DEVD-FMK, zVAD-FMK were purchased from Enzyme Systems Products. Chromotography media were obtained from Pharmacia. Recombinant caspase-3 was expressed in E. coli, purified, and stored in 50% glycerol, 10 mM Hepes (pH7.5), 1 mM EDTA, 25 mM DTT.

In Vitro Apoptosis Assay

Samples to be assayed were added to an assay mixture containing 10 mM Hepes pH 7.4, 50 mM sodium chloride, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 0.83 mM ATP, 8.3 mM creatine phosphate, 33.2 g/ml creatine kinase, 1 g/ml CPP32 to a final volume of 20 1 and allowed to sit for 30 min at room temp. Final sodium chloride concentrations were maintained between 50-150 mM to prevent nuclei from lysing. Naive nuclei were diluted in OB buffer, 5 1 was added to each assay reaction (6.5×10^5 nculei total), and the reaction allowed to incubate at 37 C for 60 min. Column fractions containing CPP32-activated CPAN (post Poly-U Seph #2) were assayed with nuclei directly, omitting the addition of CPP32 to the assay reaction and the 30 min preincubation step. To determine the percentage of apoptotic nuclei, samples were diluted ½ with Hoechst 33258 stain and counted in a hemacytometer under a microscope viewed with visible and fluorescent light.

EXAMPLE 2

Activation of a cytosolic nuclease by caspase-3.

To determine whether caspases act directly on nuclei or indirectly through a cytosolic factor, we added recombinant caspase-3 to naive nuclei in the presence or absence of cytosol from unstimulated cells. Caspase-3 alone (Fig 2., lane 1) or cytosol alone (lane 2) did not induce DNA fragmentation. When Caspase-3 was added to cytosol however, DNA fragmentation was detected indicating the activation a cytosolic factor by caspase-3. The active factor was not inhibited by the addition of caspase inhibitor DEVD-FMK added after caspase activation (lane 4). Addition of DEVD-FMK to caspase-3 prior to addition of cytosol prevented the factor from becoming activated (lane 5). When the same experiment was repeated using naked human DNA instead of nuclei, a similar result was obtained. Caspase-3 alone (lane 6) or cytosol alone (lane 7) was not active at degrading naked DNA

Addition of caspase-3 to cytosol however, activated a inactive endonuclease resulting in the non-specific fragmentation of human DNA (lane 8). The active endonuclease was not inhibited by DEVD-FMK added after caspase activation (lane 9) but was dependent on the addition of active caspase-3 (lane 10). These results suggest that the DNA fragmentation we observed in whole nuclei was due to the activation of a cytosolic endonuclease which once activated, traffics into the nuclei to cause internucleosomal DNA fragmentation. We have named this endonuclease caspase-Activated Nuclease, or CPAN.

Preparation of Naive Nuclei

Jurkat cells were grown to 1 x 10^6 viable cells/ml, recovered by centrifugation, and washed twice in ice-cold phosphate-buffered saline. Cells were washed in hypotonic lysis buffer NB3 (10 mM PIPES, 10 mM KCl, 2 mM $MgCl_2$ pH 7.4), and then resuspended in buffer NB3 at approximately 15 x pellet volume. The cells were held on ice for 20 minutes and then ruptured by 50 strokes with a Dounce homogenizer. To the homogenate one tenth volume of 2 M sucrose in buffer NB3 was added, and the homogenate centrifuged over a sucrose pad (buffer NB3 + 2 M sucrose) at 21,000 x g for 45 minutes. The pellet (nuclei) was collected, washed in buffer NB3 and centrifuged at 500 x g. The washed nuclei were resuspended in OB buffer (50 mM Tris, 2 M sucrose, 5 mM $MgCl_2$, pH 7.5) at 2.5 x 10^8 nuclei/ml, frozen in liquid N_2, and stored at -80 C.

EXAMPLE 3

Purification of the cytosolic endonuclease CPAN

The apoptotic factor CPAN was purified by fractionating unactivated Jurkat cytosol and then assaying for apoptotic activity following activation of fraction aliquots with caspase-3. Since our initial correlation between apoptotic and nuclease activity in unfractionated cytosol was not conclusive, we chose to use the in vitro apoptosis assay during the purification to identify active fractions. Apoptotic activity was quantitated microscopically following Hoechst staining of nuclei. Cytosol from sixty-five liters Jurkat cells was fractionated by cation exchange chromatography on an SP-Sepharose column. A major peak of apoptotic activity was detected in the in vitro apoptosis assay. Fractions enriched for the factor were pooled and subjected to preparative molecular sizing chromatography. A single peak of the apoptotic activity was detected eluting at a molecular weight of 130 kD.

To purify further the apoptotic factor, we investigated a series of affinity resins for selective binding of either the unactivated or activated form of CPAN. Poly-U Sepharose was found to selectively bind the activated form of CPAN at physiologic salt concentrations while the unactivated form was unbound. This method was developed for use as an affinity purification step on a preparative scale. Non-specific Poly-U binding proteins were first removed from the preparation by passing the material through a Poly-U column and collecting the unbound fraction. The conditions for caspase-3 activation of the entire preparation were evaluated on an analytical scale with the goal of minimizing the total caspase-3 required and maximizing the yield of apoptotic activity. Increasing concentrations of caspase-3 were added and allowed to incubate for four or sixteen hours at 20 C and then assayed for in vitro apoptosis activity and nuclease activity. For both incubation times, increasing concentrations of caspase-3 lead to increased apoptotic and nuclease activity, although significantly less caspase was required to reach maximal activity with sixteen hour incubation (data not shown). Based on the conditions identified here, the entire CPAN preparation was activated with caspase-3, loaded onto a Poly-U column, and then eluted with a salt gradient. The majority of the contaminating protein in the preparation passed through the Poly-U column unbound (Figure 4A). Analysis of the column fractions in the in vitro apoptosis assay (Figure 4A) showed that the apoptotic activity eluted late in the gradient after most of the contaminating proteins and correlated with the elution of a peak of nuclease activity (Figure 4B). SDS-PAGE analysis (Figure 4 C) of the column fractions revealed a single 40 KD band which correlated with both nuclease and apoptotic activity.

Molecular sizing analysis of CPAN had previously shown that the unactivated form of CPAN resolved at native molecular weight of 130 kD. The activated form of CPAN however, was shown to resolve as a large molecular weight aggregate eluting in the void volume. The explanation for the large molecular weight shift following activation remains unclear. We took advantage of this molecular weight shift to further remove contaminating proteins. The Poly-U purified, activated form of CPAN was resolved on an Sephacryl S-200 column and fractions assayed for in vitro apoptosis (Fig. 5A) and nuclease activity (Fig. 5B). A single peak of both apoptotic and nuclease activity was detected eluting in the void volume of the column. Similar to the results of the Poly-U column, both activities correlated with the intensity of a 40 KD band. Based on these results, we conclude that the single 40 KD species represents an active nuclease which is effective at inducing apoptosis in naive nuclei in vitro.

Summary of the purification of CPAN is shown in Table 1. From approximately 6 grams of starting protein, we recovered 6 g of CPAN at a purification of over 11,000-fold and a total yield of 1.2%. The Poly-U affinity column alone achieved a 300-fold purification with a stepwise yield of 46%. Although CPAN was significantly purified on the second molecular sizing step, the summary table indicates that we suffered a substantial loss in total activity with no apparent increase in specific activity. We believe this resulted from the poor stability of the active factor in a purified form which lost over 50% of the total activity in 24 h at 4 C.

Table 1. Purification of activated CPAN from Jurkat cells

Step	Fraction	Total Protein	Specific Activity	Total Activity	Recovery	Purification
		mg	units/mg	unit	%	-fold
1	Cytosol	5632	54	305250	100	1
2	S-Sepharose	440	418	183736	60	8
3	S-200 HR 1	93	1,290	119050	39	24
4	Poly-U FT 2	66	2,125	140625	46	39
5	Poly-U Eluate	0.1	625,000	64000	21	11,530
6	S-200 HR 2	0.006	625,000	3520	1.2	11,530

Cytosol from 65-liters of Jurkat cells was purified as described in Experimental Procedures. Protein concentration was determined using the BCA Protein Reagent Method (Pierce) except for the S-200 HR 2 pool in which the protein concentration was estimated based on SDS-PAGE band intensity visualized using Coomassie stain. The Poly-U FT 2 fraction indicates the S-200 HR 1 pool that had been passed through a Poly-U column two times in an unactivated form to remove non-specific Poly-U binding proteins. Units of CPAN activity were determined by measuring the apoptotic activity of serial dilutions of the purification samples in the in vitro apoptosis assay quantitated microscopically using Hoescht stain. One unit was arbitrarily defined as the amount of CPAN required to cause 50% apoptosis 6.5 x 10^5 total Jurkat nuclei in a 25 µl reaction. All unactivated purification samples were activated with caspase-3 (25ng) for 30 min at RT prior to performing the assay.

Purification of inactive CPAN from Jurkat Cytosol

Sixty-five liters of Jurkat T cells (lymphoma T-cell line, ATCC clone TIB152) were grown in RPMI 1640 supplemented with 10% FCS, 2mM L-glutamine, 100 U/ml penicillin G and 100 ug/ml streptomycin to 1.5 x 10^6 cells/ml. The cells were washed once with RPMI alone and then resuspended at 2 x 10^8 cells/ml in 10 mM Hepes, 50 mM sodium chloride, 2 mM $MgCl_2$, 10 mM EDTA, 5 mM EGTA, 200 uM PMSF and 2 ug/ml leupeptin. All subsequent cell fractionation and chromatography steps were performed at 4 C. Cells were lysed by four freeze/thaw cycles and centrifuged for 10 min. at 10,000 x g, then 100,000 x g for 30 min. The clarified cytosol was made 0.25 M sucrose and then dialyzed into 10 mM Hepes pH 7.4, 0.25M sucrose, 2mM $MgCl_2$, 5 mM EGTA, 5 mM EDTA, 2 mM DTT, 200 uM PMSF and 2 ug/ml leupeptin. Following dialysis the cytosol was adjusted to pH 7.2, reclarified by centrifugation at 100,000 x g for 30 min and loaded onto a SP Sepharose HP column (2.6 x 25 cm). The protein was eluted using a 330 ml 0-0.6 M sodium chloride gradient. Peak fractions containing in vitro apoptosis activity were identified using the in vitro apoptosis assay. Highly active fractions were pooled and subjected to size exclusion chromatography on an Sephacryl S-200 column (5 x 85 cm) in two runs (34 ml load each) in 10 mM Hepes pH 7.4, 0.25 M sucrose, 150 mM sodium chloride, 5 M EGTA, 2mM $MgCl_2$, and 2 mM DTT (SEC buffer). Fractions enriched in in vitro apoptosis activity were pooled and concentrated to 50 ml by ultrafiltration on a YM-10 membrane.

Preparative Activation and Purification of CPAN

The concentrated SEC pool was passed through a Poly-U Sepharose column (1.6 x 15 cm) equilibrated in SEC buffer. Total protein passing through the column (unbound fraction) was pooled. The protein bound to the Poly-U column was eluted with an 80 ml 0-1.5 M sodium chloride gradient in SEC buffer and the column reequilibrated in SEC buffer alone. The unbound fraction was passed through the Poly-U Sepharose column a second time to remove all poly-U binding protein. Total unbound protein passing through the column was pooled and concentrated to 45 ml by ultrafiltration on a YM-10 membrane. To preparatively activate CPAN, recombinant CPP32 was added to 128 ng/ml, fresh DTT to 4 mM, and the digest allowed to incubate for 16 h at 20 C. The activated CPAN was loaded onto a second Poly-U Sepharose column (1.6 x 15 cm) equilibrated in SEC buffer, washed until no protein was detected in the eluate, and then eluted with a 80 ml 0-1.5 M sodium chloride gradient in SEC buffer. Fractions were analyzed for in vitro apoptosis activity, nuclease activity, and by SDS-PAGE. Fractions enriched in apoptotic activity were pooled, concentrate by ultrafiltration on YM-10 membrane to 0.5 ml, and resolved by size exclusion chromatography on a Sephacryl S-200 column (0.9 x 33 cm) equilibrated in SEC buffer. Fractions were analyzed for in vitro apoptosis activity, nuclease activity, and by SDS-PAGE. Fractions enriched in apoptotic activity were pooled, concentrated by ultrafiltration on YM-10 membrane to 0.5 ml, and resolved by size exclusion chromatography on a Sephacryl S-200 column (0.9 x 33 cm) equilibrated in SEC buffer. Fractions were analyzed for in vitro apoptosis activity, nuclease activity, and by SDS-PAGE. CPAN was alkylated with further purified by preparative SDS-PAGE, electroeluted and concentrated using an Amicon Centrilutor. Sequence of N-terminal and internal peptides of CPAN were obtained.

Nuclease Assay

Column fractions (4 ul or dilutions thereof) were added to 4 ul of nuclease buffer (10 mM Hepes pH 7.4, 4 mM $MgCl_2$, 4 mM DTT, 4 mM EGTA, 2 mg/ml BSA (DNase free) and 50 mM sodium chloride. The sodium chloride concentration in the buffer was adjusted in an attempt to reach 150 mM final in all the samples. Human genomic DNA (4 ul, approx. 1 g, Promega #G304a in 10 mM Tris-HCL, pH 8.0, 1 mM EDTA) was added and the samples allowed to incubate for 1 h at 37 C and then analyzed by electrophoresis on 1% agarose gels visualized with ethidium bromide staining.

Alternative Nuclease Assays

Alternative assays for CPAN activity include using ^{3}H-DNA as a substrate and measuring the production of TCA soluble ^{3}H-nucleotides. A second, more useful assay is a fluorescence assay. To do this we synthesize a double-stranded fluorescently labeled probe with biotin on one end and the fluorescent group or radioactive ^{32}P on the other. The nuclease reaction takes place in solution, adding active CPAN plus or minus an inhibitory compound. After incubating the nuclease with the substrate, the biotin labeled probe is bound by avidin coated on a plate. Any cleaved probe has the fluorescent group or ^{32}P group remaining in solution no longer bound to the plate by biotin-avidin interaction. The unbound fraction is then measured in a fluorescent plate reader or by scintillation counting to detect active or inhibited CPAN. It is preferred that the substrate be bound to the plate prior to adding CPAN to lower background. Using this assay in a high throughput format allows useful screening of potential inhibitory and activating compounds.

EXAMPLE 4

Cloning of the cDNA for CPAN

The 40 KD protein band was further purified by preparative SDS-PAGE and subjected to sequence analysis. Amino-terminal sequence data was obtained through the first thirty residues. Internal sequence data was derived from six peptides generated by either Lys-c or clostrapain digestion. A total of 161 residues of CPAN were identified (underlined in Fig 6). Search of the public Expressed Sequence Tag (EST) data bases failed to identify a EST corresponding to sequences of CPAN.

To isolate the gene for CPAN, degenerate oligonucleotides encoding the amino- and carboxy-terminal ends of peptide IV (underlined in Figure 6) were used to amplify using Polymerase Chain Reaction (PCR) a 70 bp DNA fragment of the predicted size from a human placenta library. The unique DNA sequence obtained from the center of the PCR product between the two degenerate probes was used to do nested PCR which identified a 400 bp fragment encoding peptides IV, V, and VI. Using the 400 bp fragment as a probe, three million clones from a bone marrow library were screened. Two cDNA clones of CPAN were identified, only one of which (clone 11) was full length encoding the N-terminal sequence of CPAN. Clone 11 was 3.3 kb in length. Comparison of the protein reading frames to the CPAN peptide sequences revealed that clone 11 contained two apparent insertions in the 5' coding region introducing a frameshift and a frameshift and a stop codon, respectively.

To determine the correct coding sequence, PCR probes were used to amplify across the region of the insertions using a variety of human cDNA libraries. The bone marrow library was found to contained only the long form of CPAN with the two inserts. Human leukocyte, testis and Hela libraries produced both a long form and a shorter form of approximately 100 bp less. Placenta, heart and spleen libraries contained only the short form of CPAN. Sequence obtained for the short form of CPAN from leukocyte, heart and Hela cells were identical and encoded an open reading frame free of insertions. Based on these sequence data, the bone marrow clone was repaired to encode a single open reading frame of 338 amino acids. Comparison of the repaired bone marrow clone to full length short clones obtained from human leukocyte, pancreas, and Hela cell indicated that the clones are identical except for a single change of lysine to an arginine at position 197. Such a conservative change is consistent with allelic variation. Analysis of long clones of CPAN from leukocyte and Hela libraries revealed that each clone contained two inserts similar to the bone marrow clone, although the nucleotide sequences of inserts were slightly different. In both cases however, the inserts introduced frame shifts or stop codons thus preventing the generation of an extended full-length open reading frame from any of the long clones we obtained.

The amino acid sequence predicted from the CPAN cDNA clone (Figure 6) encodes a basic protein (pI 9.6) with a predicted molecular weight of 39,078 daltons. A search of the protein and nucleotide data bases indicated that CPAN is a novel protein. In addition, CPAN was also found to have no significant homology to known nuclease protein families based on results of Smith-Waterman analysis and profile homology searching (Birney et al., 1996, Henikoff et al., 1996). Northern blot analysis with the CPAN cDNA of human tissues is shown in Figure 7. A 3.5 kb mRNA band was detected in all tissues examined. A large molecular weight band (>10 kb) was also detected in several tissues. Northern blot analysis of eight human cancer cell lines indicated that CPAN is more abundant in promyelocytic leukemia (HL-60), a colorectal carcinoma (SW480), and a melanoma (G361), but was almost undetectable in Burkitt's lymphoma (Raji) and lung carcinoma (A549) (Fig. 7B).

Cloning

The gene for CPAN was isolated in three steps. The first specific nucleotide sequence was isolated by PCR from a placenta cDNA library (Clontech Marathon-Ready) using degenerate oligonucleotide primers 5'-GCNTTYCAYGARCCICARGTIGG-3' and 3' primer 5'-TGIGGIGCYTGYTCRTCRCA-3' across the peptide fragment

AFHEPQVGLIQAAQQLLCDEQAPQ. Inosine was combined with cytosine at residues where inosine is indicated. The correct product, a 72 bp fragment, was identified by size and confirmed by PCR nesting, purified from a 10% THE-acrylamide gel, and cloned into TA cloning vector pCR2.1 (Invitrogen) for DNA sequencing, resulting in a specific 32 bp sequence.

The second specific fragment was isolated from the same library by nested PCR using the specific sequence. The primary reaction was RACE with a 5' half-specific primer 5'-GARCCICARGTIGGGCTCATCCAG-3' and the library's 3' adaptor primer, AP1, 5'-CCATCCTAATACGACTCACTATAGGGC-3'. The resulting template was nested using specific 5' primer 5'-CCAGGCCGCCCAGCAGCTGCTGTG-3', and degenerate 3' primer 5'-TCCATRTCRAAIGGICCYTGRCA-3' to another peptide sequence, CWGPFDMD. Electrophoresis of the products indicated a ~400 bp band which was subsequently cloned and sequenced. Sequence encoding flanking peptide residues as well as a third internal peptide sequence contained within confirmed its identity with the CPAN gene. The 400 bp fragment was amplified from the vector, agarose gel-purified and labeled with ^{32}P using an Amersham Rediprime kit. The labeled probe was used to screen a bone-marrow library prepared in a ZapExpress lgt10 phage plated on XL1-MRF' Blue cells.

EXAMPLE 5

Expression of CPAN in association with DFF45

The cDNA for CPAN was engineered with a tag at its amino terminus and inserted into a mammalian expression vector behind the CMV promoter. To determine whether CPAN is associated with a recently discovered DNA fragmentation factor, DFF45, we used PCR to obtain the cDNA for DFF45 from a human liver library and then inserted it into a similar mammalian expression vector but with a GluGlu tag at the amino terminus of DFF45. Both expression vectors were transfected into CHO cells either separately or in combination. CPAN alone (Fig. 8A, lane 1) was expressed poorly in cells and was recovered only when cells were extracted with SDS-lysis buffer. Extraction with the detergent Triton X-100 resulted in no detectable CPAN being recovered (lane 3). When CPAN was expressed in the presence of DFF45 (lane 4) however, CPAN was expressed at higher levels and was recovered in a mild detergent buffer. Expression of DFF45 alone (Fig. 8B, lanes 1 and 3) resulted in a high yield of DFF45 in the presence or absence of SDS. In addition, the expression levels of DFF45 remained relatively constant in the presence or absence of

CPAN. These results suggest that CPAN is dependent on the presence of DFF45 to be stable in cells while DFF45 stability is independent of CPAN.

To demonstrate the association of CPAN with DFF45, we immunoprecipitated DFF45 using the GluGlu antibody and then did Western blot analysis using the anti-HA antibody to CPAN. As shown in Fig. 8A, lane 8, CPAN was quantitatively recovered from the CHO lysate associated with DFF45. An unrelated protein (AKT) was also co-expressed with DFF45 and subjected to the same immunprecipitation. No detectable AKT was recovered with the DFF45 indicating that the association between DFF45 and CPAN is specific. Immunoprecipitation of CPAN using the anti-HA antibody followed by Western analysis using an antipeptide antibody to the C-terminus of DFF45 revealed similar results. DFF45 was quantitatively recovered from the CHO extract associated with CPAN while the control protein AKT bound no detectable DFF45. These results suggest that the majority of the CPAN and DFF45 in the co-transfected CHO cells are associated in a complex.

Native CPAN purified from Jurkat cytosol was active as a nuclease only after treatment with caspase-3. To determine whether recombinant CPAN from CHO cells was active, we immunoprecipitated the CPAN from the transfections described above with an anti-HA antibody and then assayed for nuclease activity with and without caspase-3 treatment. As shown in Figure 8C, lanes 1-5, none of the samples were active prior to caspase-3 treatment. After caspase-3 treatment, however, the CPAN/DFF45 sample displayed nuclease activity and cleaved naked human DNA.

Western blot analysis of CPAN and DFF45 before and after caspase treatment (Fig. 8D) reveal that CPAN is resistant to caspase-3 treatment while DFF45 is degraded into 11 K fragments. Only a single fragment of DFF45 is detectable since the antibody used is to the C-terminus of DFF45. These results are consistent with published data (Lui et al., 1997) showing that DFF45 is cleaved at two caspase cleavage sites in apoptotic cells. The CPAN protein sequence however, does not contain any consensus caspase cleavage sites. Our results suggest that when CPAN is expressed, it is dependent on association with DFF45 to achieve a stable and biologically competent conformation. By associating with DFF45, however, it is also inhibited as a nuclease. Only upon cleavage of DFF45 by caspases does CPAN become active as a nuclease.

cDNA Cloning and Expression of DFF45

The full length DFF45 cDNA was PCR amplified from a human liver cDNA library (Gibco, BRL) using the primers 5' cgaattcgatctagaatggaggtgaccggggacgccggggtaccagaatctggcgaga and 3' cccaagcttcccgggctattccattggcatgtattctgtgggatcctgtctggctcgcttaggattctgcaggtca, which included an in-frame Glu-Glu tag at the C-terminus. The Glu-tagged DFF45 cDNA insert was then subcloned into the XbaI/PspA1 sites of the mammalian expression vector pCG or in-frame into the XbaI/HindIII sites of pCGG (pCG modified to include a Glu-Glu tag as an N-terminal fusion). Each caspase cleavage motif (DXXD) within DFF45 was mutated at both P1 and P4 positions (from D to A) to generate mutants that could not be cleaved by caspases during apoptosis. These mutants were generated using the Quick-change PCR mutagenesis kit (Stratagene) and the primers 5' caagagtcctttgatgtagctgaaacagccagcggggc and 3' gccccgctggctgtttcagctacatcaaaggactcttg for DFF114/117A, primers 5' ggtgaggaggtggctgcagtagccacggg and 3' ctgctgatacccgtggctactgcagccacctcctcaccaaag for DFF221/224A or a combination of all four primers for generation of the DFFQM construct. The nucleotide sequence of all PCR generated constructs was confirmed by sequencing analysis.

Expression of CPAN and DFF45 in insect cells

The sequence encoding c-terminal KT3 tagged CPAN was inserted into the pFASTBAC donor plasmid (GIBCO BRL) downstream of the polyhedrin promoter. Recombinant bacmid was subsequently isolated following site-specific transposition to the Tn7 attachment site in DH10BAC E.coli (GIBCO BRL) following transformation of these cells with the pFASTBAC vector. Bacmid DNA was isolated and amplified as described (Luckow et al., 1993). The sequence encoding C-terminal Glu tagged DFF45 was subcloned into the Autographa californica baculovirus via the BacPAK-His2 transfer vector (Clontech). Recombinant baculovirus was generated by homologous recombination (Kitts et al., 1990) and isolated by plaque purification *(Smith et al., 1983)*. Suspension cultures of 1.5x10e6 Sf9 cells per ml were infected with the relevant baculovirus at moi of 1-5 for 72 hours at 25 C in serum-free media *(Maiorella et al., 1988)*.

EXAMPLE 6

CPAN is activated in apoptosis

The association of CPAN with a caspase-sensitive inhibitor suggests that when cells enter into apoptosis, the activation of caspases will trigger CPAN to become active. To determine whether this occurs, we transfected CHO cells with the CPAN and DFF45 expression constructs and 24 h later induced apoptosis with staurosporine. Cell extracts were prepared at various time points and assayed for caspase activity and for activation of CPAN as measured in a nuclease assay following immunprecipitation with the anti-Flag antibody. Within 1 hour after induction of apoptosis, caspase activity began to increase and was maximal by the 6 hour time point (Fig. 9A). Caspase levels were similar in cells transfected with CPAN and DFF45 or DFF45 alone. Western blot analysis of CPAN and DFF during the apoptotic time course indicate that CPAN remains intact in apoptotic cells while DFF45 is degraded into 11 K fragments, although only one fragment is detected here. A small amount of the 11 K DFF45 fragment is seen prior to induction of apoptosis which may derive from cells that have spontaneously entered apoptosis due to low level toxicity in transient transfections. CPAN and DFF45 were separately immunoprecipitated with tag antibodies and then assayed in the nuclease assay. CPAN nuclease activity (Fig. 9B) increased in direct correlation with the increase in caspase activity, reaching maximal levels by four hours. When DFF45 was tested in the same assay, no nuclease activity was detected. Our results indicate that prior to an apoptotic stimulus, CPAN exists in the cell in an inactive form associated with intact DFF45. Upon activation of apoptosis and the cysteine protease cascade, DFF45 in complex with CPAN is cleaved at one or both of its caspase cleavage sites allowing CPAN to be activated as a nuclease.

EXAMPLE 7

Disassociation of DFF45 fragments from activated CPAN

Association of CPAN with DFF45 appears essential for CPAN to achieve a stable and yet inactive conformation in the cell. It is unclear however, whether subsequent to caspase activation the fragments of DFF45 remain associated with CPAN and are required for nuclease activity.

To examine this, we co-expressed CPAN and DFF45 in insect cells. Similar to the CHO cell results, expression of KT3-tagged CPAN alone in insect cells failed to produce any detectable protein by Western blot (data not shown). Inthe presence of DFF45, however, significant quantities of CPAN accumulated inthe cell. Purification of CPAN/DFF45 complex using an anti-KT3 antibody column produced a purified protein complex which resolved as two bands by SDS-PAGE (Fig 10A, lane 1). The 43.5kD band was identified as DFF45 by anti-DFF45 immunoblotting, and the 40kD band was identified as CPAN by anti-KT3 immunoblotting (data not shown). The purified CPAN/DFF45 complex was activatedby treatment with caspase-3. DFF45 was cleaved by caspase-3 at two sites, generating three peptide fragments of molecular weight 16kDa, 12kDa, and 10kDa (Fig 10A, lane 2), consistent with published data on the DFF complex (Lui et al., 1997). CPAN was not cleaved by caspase-3 treatment (lane 2). The activated CPAN/DFF45 complex was purified by Poly-U chromatography. Fragments of DFF45 were not bound by the Poly-U resin and flowed through the column (lane 3). CPAN, however, was selectively bound by the resin and eluted in a highly purified form, free of any associated DFF45 fragments or other insect cell contaminants (lane 4).

To assess the activity of CPAN during this procedure, we measured in vitro apoptosis using naive Jurkat cell nuclei. Prior to caspase-3 activation, the purified insect cell-derived CPAN/DFF45 complex was inactive (Fig 10B, lane 1). Following caspase-3 treatment, the complex was active at degrading nuclear chromatin (lane 2). When the activated complex was purified on the Poly-U column, essentially all of the nuclease activity bound to the Poly-U column (lane 3) and eluted with the purified CPAN protein (lane4). These results indicate that caspase-3 activates the DFF45/CPAN complex by cleaving DFF45 and releasing DFF45 fragments from CPAN.

EXAMPLE 8

CPAN nuclease activity is inhibited by intact DFF45.

Once the CPAN/DFF45 complex is cleaved by caspases, the fragments of DFF45 dissociate and CPAN becomes activated as a nuclease. To determine whether intact DFF45 can bind to and inhibit the activated form of CPAN, we added recombinant human DFF45 produced in insect cells to the active, Poly-U purified CPAN from the previous experiment and then measured for inhibition of CPAN activity in the nuclease assay and in the in vitro apoptosis assay. Activated, purified CPAN alone was highly active in either the nuclease

assay or in the in vitro apoptosis assay. Addition of increasing concentrations of DFF45 to CPAN inhibited its activity in a dose dependent manner in both assays. Addition of caspase-3 to the DFF45-inhibited CPAN sample at the highest concentration of DFF45 fully restored the activity of CPAN demonstrating again that the inhibition of CPAN by DFF45 is caspase-sensitive. The amount of intact DFF45 required to inhibit CPAN was significantly greater than equimolar suggesting that the activated form of CPAN has a lower affinity for DFF45 than does the nascent CPAN polypeptide.

Expression of CPAN and DFF45 in insect cells

The sequence encoding c-terminal KT3 tagged CPAN was inserted into the pFASTBAC donor plasmid (GIBCO BRL) downstream of the polyhedrin promoter. Recombinant bacmid was subsequently isolated following site-specific transposition to the Tn7 attachment site in DH10BAC *E. coli* (GIBCO BRL) following transformation of these cells with the pFASTBAC vector. Bacmid DNA was isolated and amplified as described (Luckow et al., 1993). The sequence encoding C-terminal Glu tagged DFF45 was subcloned into the Autographa californica baculovirus via the BacPAK-His2 transfer vector (Clontech). Recombinant baculovirus was generated by homologous recombination (Kitts et al., 1990) and isolated by plaque purification *(Smith et al., 1983)*. Suspension cultures of 1.5x10e6 Sf9 cells per ml were infected with the relevant baculovirus at moi of 1-5 for 72 hours at 25 C in serum-free media *(Maiorella et al., 1988)*.

Purification and characterization of recombinant CPAN

The KT3-CPAN baculovirus was co-infected with the 6XHIS-DFF45-GluGlu baculovirus in 600 ml of insect cells and harvested at 72 hr. 6XHIS-DFF45-GluGlu baculovirus was infected alone in 500ml of insect cells and harvested at 72 hr. The cells from both cultures were solubilized in buffer C containing 1% Triton X-100 and clarified by centrifugation (10k x g for 10 min at 4 C). The clarified lysate from the CPAN/DFF45 co-infection was purified on a KT3 monoclonal antibody covalently coupled to Protein G Sepharose (3 ml). The unbound fraction was collected, the column washed in buffer C lacking added Triton X-100, and the purified CPAN eluted with a KT3 peptide (100μg/mL) and concentrated by ultrafiltration to 1.7 ml. To activate CPAN, caspase-3 (2μg) and DTT (2 mM) were added and allowed to incubate for 3.5 hr at 20°C. The activated CPAN was loaded onto a Poly-U Sepharose column (1.6 x 10 cm) equilibated in buffer B and then eluted

with a 0-1M sodium chloride gradient as a single protein peak. An aliquot of the pool (500ul) was dialyzed into 5mM sodium phosphate buffer (pH 7.0) containing 0.1% SDS and 1 mM DTT, lyophilized, and subjected to SDS-PAGE on a 10-20% gradient Tricine Gel. To stabilize the remaining pool, BSA was added to 0.2 mg/ml and the pool stored at 4 C. The clarified lysate from the culture infected with DFF45 alone was passed over a nickel-agarose column (Qiagen), washed, and eluted in buffer A containing 2 mM DTT with 200 mM imidazole. Purified DFF45 was stored at -20 C at 1.35 mg/ml. To determine the apoptotic activity of the purified CPAN samples. The purified CPAN/DFF45 complex (0.32ul), the activated complex (0.32ul), the Poly-U fall through fraction (3.2ul), and the active CPAN Poly-U eluate (3.2ul) were tested in the in vitro apoptosis assay for 2 hr at 37°C at a final sodium chloride concentration of 75 mM. Genomic DNA was extracted from the assay samples and analyzed by 1% agarose gel ectrophoresis. To inhibit the active CPAN eluate (3.2ul, 12.8 ng), purified DFF45 (100ng or 33ng in duplicate) or ZVAD-FMK, DEVD-FMK (25μM final each) were added to the assay mixture containing active CPAN and incubated for 15min at RT. Caspase-3 was then added to two of the four DFF45 samples, incubated for 45 min at RT, and the samples assayed as described above. Nuclease activity of the purified CPAN samples was also determined using a 14μl assay containing one half sample volumes described above. Samples were incubated with 0.8μg of naked DNA for 10 min at 30°C and then analyzed by 2% agarose gel electrophoresis.

EXAMPLE 9

Mutation of DFF45 caspase cleavage site blocks CPAN activation

DFF45 contains two caspase-3 cleavage sites that are both cleaved in apoptotic cells (Lui et al., 1997). We have engineered three DFF45 mutants in which the two caspase cleavage sites have been mutated separately or in combination by changing the aspartic acid in the P1 and P4 position to alanine. All three of the DFF45 constructs were engineered with a GluGlu tag at both the N- and C-termini. The DFF45 mutants and wildtype DFF45 were separately co-transfected with CPAN and after 24 hours apoptosis was induced. In this experiment, we induced apoptosis through the Fas receptor by using a CHO cell line stably expressing a chimera of the cytoplasmic domain of the murine Fas antigen fused to the extracellular domain of the human CD4 receptor (Lee et al., 1997). This cell line was used to study Fas-induced cell death instead of using Jurkat cells because of the poor transfection efficiency typically observed with Jurkat cells. Crosslinking of the

CD4 receptor with an anti-CD4 monclonal antibody rapidly induced apoptosis and the appearance of caspase activity within 15 min. Cells were harvested just prior to and two hours post Fas activation using the CD4 antibody.

Western blot analysis using the anti-GluGlu antibody of wildtype DFF45 co-transfected with CPAN shows that following Fas activation, DFF45 was cleaved into several products, an intermediate cleavage products at 23kDa and two smaller cleavage products at 18kDa and 11kDa representing the N- and C-terminal fragments of DFF45 containing the GluGlu tags. Fas activation of the single DFF45 mutant Asp-114 to Ala, Asp-117 to Ala ($DA^{114}DA^{117}$) in which the first caspase cleavage site has been mutated was degraded by caspases to two products of 11kDa and 30 kDa representing the N-terminal fragment (11kDa) and the C-terminal two thirds of DFF45 (35kDa). Induction of apoptosis with the second DFF45 mutant Asp-221 to Ala, Asp-224 ($DA^{221}DA^{224}$) in which the second caspase cleavage site was mutated produced different cleavage products, a 23kDa species comprising the N-terminal two thirds of DFF and an 18kDa species containing the C-terminus of DFF45. Analysis of the double mutant of DFF45, in which both sites were altered, showed no proteolysis following induction of apoptosis indicating it was resistant to caspase cleavage.

To examine the effect of these mutants on CPAN activation, we immunoprecipitated CPAN from each of the transfections to measure nuclease activity and examine the DFF45 species associated with CPAN. Prior to Fas activation of apoptosis, all of the DFF45 constructs were detected in a complex with CPAN as intact 45kDa protein species. Induction of apoptosis with wildtype DFF45 lead to the disassociation of the caspase cleavage products, consistent with previous data. Analysis of the cleavage products associated with CPAN for the single site mutant $DA^{114}DA^{117}$ revealed that the large cleavage product containing the last two thirds (30kDa) of DFF45 remained associated with CPAN while the small N-terminal fragment disassociated. The large cleavage product of the second site mutant $DA^{221}DA^{224}$ also remained associated with CPAN while the small N-terminal fragment dissassociated from CPAN. The double site mutant was not cleaved by caspases and remained tightly associated with CPAN. The double site mutant was not cleaved by caspases and remained tightly associated with CPAN. A constant amount of CPAN was immunoprecipitated in each of the transfections examined here. These results suggest that the region between the cleavage sites is necessary for association with CPAN. However, this region is not sufficient for association

since no fragments of DFF45 are associated with CPAN after cleavage of wild type DFF45 at both sites.

Induction of apoptosis in cells transfected with CPAN and wildtype DFF45 lead to the actiavtion of CPAN as a nuclease. In cells co-transfected with the first DFF45 clavage site mutant $DA^{114}DA^{117}$, induction of apoptosis failed to activate CPAN, presumably due to the remaining association of the uncleavable DFF45 fragment containing the first two thirds of DFF45. With the second site DFF45 mutant $DA^{221}DA^{224}$, induction of apoptosis resulted in the activation of CPAN which indicates that association of the last two thirds of DFF45 with CPAN was not sufficient to block the activity of CPAN. Activation of apoptosis in cells transfected with CPAN and the double cleavage site mutant completely prevented the activation of CPAN consistent with results of the individual mutants.

Transient expression in CHO cells

CHO cells were transfected with either CPAN (3ug) or DFF45 (3ug) or both using LT1 as described below. Total DNA added was kept constant by adding empty vector. In a control transfection, DFF45 was co-transfected with an AKT expression vector (3ug) which contained the complete AKT open reading frame fused into the pCGN vector with an HA epitope at the amino-terminus. Cells (aproximately 70-90% confluent on 10 cm plate) were washed with OPTIMEM™ medium (Gibco BRL), transfected for 5 hr with LT1 (30ul):DNA mixture in 4 ml of OPTIMEM™ medium , and the OPTIMEM™ medium then replaced with fresh medium. After 24 hr, the cells were either harvested for analysis or induced into apoptosis with staurosporine (1 μM) and harvested at the indicated times. Cells were harvested, recovered by centrifugation (1000 x g for 10 min at 4 C) and resuspended in 100μl of buffer C (buffer A containing 150 mM sodium chloride [final], 200 μM PMSF, and 2 μg/ml leupeptin). For caspase assay, the cells (10μl) were lysed by dilution into 200ul of buffer C containing 1% Triton X-100 and 2 mM DTT, clarified by centrifugation (10k x g for 2 min at 4 C) and assayed in a fluorometer using ZDEVD-AFC (25μM). For Western blot analysis, the cells (10ul) were lysed by addition of buffer C (10 μl) containing 2% Triton X-100, 2 mM DTT, and 20 μM ZVAD-FMK, clarified by centrifugation, and subjected to 10% SDS-PAGE. For protein analysis and nuclease assay, the cells (10μl) were lysed by addition of buffer C (400 μl) containing 1% Triton X-100, 2 mM DTT, and 20 μM ZVAD-FMK, clarified by centrifugation, and then immunoprecipitated for Western blott analysis and measurment of nuclease activity. CPAN

and DFF45 were also transfected into CHO cells stabily expressing a chimera of the extracellular domain of CD4 fused to the cytoplasmic domain of murine Fas antigen (Lee et al., 1997) and after 24 hr induced into apoptosis using an anti-CD4 antibody (x μg/ml). After 75 min the cells were washed with phosphate-buffered saline and then lysed with 200 μl of 10 mM Tris, 137 mM sodium chloride, 1% Triton X-100 15 % glycerol containing 20μM ZVAD-FMK, 10 μg/ml of aprotinin, leupeptin, pepstatin. The extract was then clarified by centrifugation and immunoprecipitated (20μl) for Western blot analysis and measurement of nuclease activity.

Immunoprecipitation and Western blot analysis

Clarified extracts were immunoprecipitated with the appropriate monoclonal antibody (1μg) for 2 hr at 4 C and then bound to Protein-A Sepharose (20μl) for 1 hr at 4 C. Samples were washed (3x) with extract buffer and then either assayed directly in the nuclease assay or eluted with non-reducing SDS-PAGE sample buffer containing 20 mM iodoacetamide and subjected to SDS-PAGE using either 10% or 16% tris-glycine gels (Novex). For the immunoprecipitation of native DFF45/CPAN complex (Figure 5A), native CPAN purified through the S-Sepharose step (100μl) was immunoprecipitated by 1/5 dilution into buffer C containing 0.5% Triton X-100 and 10μg of affinity purified anti-C-terminal peptide antibody to DFF45, incubated for 2 hr at 4 C, and then bound to 20μl of Protein-G Sepharose (1 hr at 4 C). The beads were washed 3x, and then eluted in 40μl of buffer C containing either the C-terminal DFF45 peptide (amino acids 314-331, 250 μg/ml) or the N-terminal CPAN peptide (amino acids 1-25, 250μg/ml) and subjected to Western blot analysis. SDS polyacrylamide gels were transferred to PVDF, blocked with Western buffer (10 mM Tris [pH 7.5],150 mM NaCl, 0.1% [vol/vol] Tween 20, 5% [wt/vol]dried milk protein), probed overnight with either anti-Flag (Upstate BioTech), anti-HA (Babco), or anti-GluGlu monoclonal antibody (10 μg/ml) or antipeptide polyclonal antibody to either the N-terminus of CPAN (residues 1-25) or to the C-terminus of DFF45 (residues 314-331), at a 1/2000 dilution. The bound antibody was detected with either an anti-rabbit or an anti-mouse antibody conjugated to HRP (1 hr, 1/20k dilution) and detected by enhanced chemiluminescence (Amersham).

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SEQUENCE LISTING

CPAN NUCLEIC ACID SEQUENCE

```
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  61 gtggctggcc ggagctgcca ggaggtgctg cgcaagggct gtctccgctt ccagctccct
 121 gagcgcggtt cccggctgtg cctgtacgag gatggcacgg agctgacgga agattacttc
 181 cccagtgttc ccgacaacgc cgagctggtg ctgctcacct tgggccaggc ctggcagggc
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 901 ctagtgcaca ttgtctgcca taagaaaacc acccacaagc tcaactgtga cccgagcaga
 961 atctacaaac cccagacaag gttgaagcgg aagcagcctg tgcggaaacg ccagtga
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CPAN AMINO ACID SEQUENCE

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101 QAAQQLLCDE QAPQRQRLLA DLLHNVSQNI AAETRAEDPP WFEGLESRFQ

151 SKSGYLRYSC ESRIRSYLRE VSSYPSTVGA EAQEEFLRVL GSMCQKLRSM

201 QYNGSYFDRG AKGGSRLCTP EGWFSCQGPF DMDSCLSRHS INPYSNRESR

251 ILFSTWNLDH IIEKKRTIIP TLVEAIKEQD GREVDWEYFY GLLFTSENLK

301 LVHIVCHKKT THKLNCDPSR IYKPQTRLKR KQPVRKRQ
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DFF45 NUCLEOTIDE SEQUENCE

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       61 aggtgaccgg ggacgccggg gtaccagaat ctggcgagat ccggactcta aagccgtgtc
      121 tgctgcgccg caactacagc cgcgaacagc acggcgtggc cgcctcctgc ctcgaagacc
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      961 gcttgcattc tctccggagc atctcagcaa gcaaggcctc accacctggt gacctgcaga
     1021 atcctaagcg agccagacag gatcccacat agcagcagcg ggaagtgtgc caaggaagct
     1081 ctgtggcgtt gtgttattgg tagacaccct cagcctcatc atttgactac ctatgtacta
     1141 ctctaccccc tgccttagag caccttccag agaagctatt ccaggtctca acatacgccg
     1201 ttccaccaat ttttttttta gccccaccag cttcaggact tctgccaatt ttgaatgata
     1261 tagctgcacc aacaatatcc cgcctcctct aattacatat gatgttctct gttcaaaagt
     1321 aattggcagt gattggccag gcgcagtggc tcacgcctgt aatcccagca ctgggaggcc
     1381 gaggggggcg gatcgtgaag tcaggagatc gagaccatcc tggctaacat ggtgaaaccc
     1441 tgtctctact aaaaatacaa aaaaaattag ccagccatgg tggcgggcgc ctgtaatccc
     1501 agctacttgg gaggctgagg caggagaatg gcatgaacct gggaggcaga gcttgcagtg
     1561 agctgagatt gcgccactgc actccagcct gggcaacaga gcgagactcc gtctcaaaaa
     1621 aaaaaaaaaa aaa
```

DFF45 AMINO ACID SEQUENCE

```
                     /note="DFF-45"
                     /codon_start=1
                     /product="DNA fragmentation factor-45"
                     /db_xref="PID:g2065561"

/translation="MEVTGDAGVPESGEIRTLKPCLLRRNYSREQHGVAASCLEDLRS

KACDILAIDKSLTPVTLVLAEDGTIVDDDDYFLCLPSNTKFVALASNEKWAYNNSDGG

TAWISQESFDVDETDSGAGLKWKNVARQLKEDLSSIILLSEEDLQMLVDAPCSDLAQE

LRQSCATVQRLQHTLQQVLDQREEVRQSKQLLQLYLQALEKEGSLLSKQEESKAAFGE

EVDAVDTGISRETSSDVALASHILTALREKQAPELSLSSQDLELVTKEDPKALAVALN
                     WDIKKTETVQEACERELALRLQQTQSLHSLRSISASKASPPGDLQNPKRARQDPT"
```

CLAIMS

1. An isolated subgenomic DNA molecule which encodes an active CPAN protein of approximately 40 kd as measured on SDS-polyacrylamide gel electrophoretograms and has an amino acid sequence as shown in SEQ ID NO: 2, or a biologically active variant thereof.

2. A nucleic acid construct comprising a promoter which is operably linked to segment of nucleic acid which encodes a 40 kd active CPAN protein as measured on SDS-polyacrylamide gel electropheretograms and has an amino acid sequence as shown in SEQ ID NO: 2, or a biologically active variant thereof.

3. The nucleic acid construct of claim 2 wherein the promoter is active in tumor cells but not in non-tumor cells.

4. An isolated subgenomic DNA molecule which encodes an immunogenic polypeptide of at least 6 contiguous amino acids of an active CPAN protein of approximately 40 kd as measured on SDS-polyacrylamide gel electropheretograms and has an amino acid sequence as shown in SEQ ID NO: 2.

5. A polynucleotide probe comprising at least 12 contiguous nucleotides selected from the sequence shown in SEQ ID NO: 1.

6. A vector comprising the isolated subgenomic DNA molecule of claim 1.

7. A vector comprising the nucleic acid construct of claim 2.

8. A vector comprising the isolated subgenomic DNA molecule of claim 4.

9. A vector comprising the polynucleotide probe of claim 5.

10. A host cell comprising the vector of claim 6.

11. A host cell comprising the vector of claim 7.

12. A host cell comprising the vector of claim 8.

13. A host cell comprising the vector of claim 9.

14. A method of digesting DNA, comprising the step of:

contacting a preparation of nuclei-free DNA with an active CPAN nuclease which is approximately 40 kd protein as measured by SDS-polyacrylamide gel electrophoresis, whereby the DNA is digested to form nucleotides and/or random DNA fragments.

15. The method of claim 14 wherein the step of contacting is performed in the presence of a carrier protein.

16. The method of claim 14 wherein the carrier is bovine serum albumin.

17. The method of claim 14 wherein the step of contacting is performed in the absence of nuclei.

18. A method of purifying an inactive CPAN having an apparent molecular weight of 130 kD as measured by size exclusion chromatography and comprising at least one subunit of approximately 40 kd as measured by SDS-polyacrylamide electrophoresis, comprising the step of:

contacting a cytoplasmic preparation with poly-uridylic acid and collecting a fraction of proteins which do not bind to poly-uridylic acid .

19. The method of claim 18 wherein the cytoplasmic preparation is first purified by cation exchange chromatography on S Sepharose HP ™ and collecting proteins from a molecular sizing column of about 130 kD.

20. A method of purifying an active CPAN protein having an apparent molecular weight of 40 kd as measured on SDS-polyacrylamide gel electrophoretograms, comprising the step of:

contacting a mixture comprising an active CPAN with polyuridylic acid, whereby the active CPAN binds to the polyuridylic acid to form a bound complex;

separating the bound complex of CPAN and polyuridylic acid from other components of the mixture.

21. The method of claim 20 further comprising the step of separating CPAN from polyuridylic acid in the bound complex by addition of an ionic salt to dissociate CPAN from polyuridylic acid.

22. The method of claim 20 wherein the polyuridylic acid is attached to a column packing matrix.

23. The method of claim 21 wherein an ionic salt gradient is applied to the bound complex, whereby the bound complex dissociates at one or more points of the gradient.

24. A method of identifying compounds which inhibit apoptosis, comprising:

contacting a test compound with an active CPAN protein having a molecular weight of approximately 40 kd as measured on SDS-polyacrylamide gel electropheretograms;

testing the CPAN for the ability to fragment DNA, wherein a test compound which inhibits CPAN fragmentation of DNA is a candidate compound for inhibiting apoptosis.

25. The method of claim 24 wherein the step of contacting is performed in the presence of isolated nucleic acids.

26. A method of identifying compounds which inhibit apoptosis, comprising:

contacting a test compound with a cell comprising a recombinant DNA construct encoding an active CPAN protein having a molecular weight of approximately 40 kd as measured on SDS-polyacrylamide gel electropheretograms;

testing the CPAN for the ability to fragment DNA, wherein a test compound which inhibits CPAN fragmentation of DNA is a candidate compound for inhibiting apoptosis.

27. A method of identifying compounds which inhibit apoptosis, comprising:

contacting a test compound with a cell comprising an active CPAN protein having a molecular weight of approximately 40 kd as measured by molecular sizing chromatography;

assaying for an apparent increase in the molecular size on molecular size exclusion chromatography of the CPAN protein in the cell, wherein a test compound which causes CPAN to behave as if it has increased in molecular size is a candidate compound for activating apoptosis.

28. A method of identifying compounds which activate apoptosis, comprising:

contacting a test compound with an inactive caspase activated nuclease (CPAN) protein having a molecular weight of approximately 130 kD as measured by molecular sizing chromatography;

testing the CPAN for the ability to fragment DNA, wherein a test compound which activates CPAN to fragment DNA is a candidate compound for activating apoptosis.

29. The method of claim 28 wherein the step of testing is performed using a nuclei-free DNA preparation as a substrate.

30. The method of claim 28 wherein the step of testing is performed in the presence of a protein carrier.

31. The method of claim 30 wherein the protein carrier is bovine serum albumin.

32. A method of identifying compounds which activate apoptosis, comprising:

contacting a test compound with an inactive CPAN protein, wherein the inactive CPAN has a molecular weight of approximately 130 kD as measured by molecular sizing chromatography;

assaying for an apparent increase in molecular size of CPAN as measured by molecular size exclusion chromatography, wherein a test compound which causes CPAN to behave as if it has a large molecular size on molecular size exclusion chromatography is a candidate compound for activating apoptosis.

33. The method of claim 32 wherein the step of contacting is performed in the presence of nucleic acids.

34. A method of identifying compounds which activate apoptosis, comprising:

contacting a test compound with a cell comprising an inactive CPAN protein having a molecular weight of approximately 130 kD as measured by molecular sizing chromatography;

assaying for an apparent increase in the molecular size on molecular size exclusion chromatography of the CPAN protein in the cell, wherein a test compound which causes CPAN to behave as if it has increased in molecular size is a candidate compound for activating apoptosis.

35. An antibody preparation which specifically binds to CPAN protein as shown in SEQ ID NO: 2.

36. A nucleic acid construct comprising a promoter which is operably linked to an antisense strand of a segment of nucleic acid which encodes a 40 kd active CPAN protein as measured on SDS-polyacrylamide gel electropheretograms and has an amino acid sequence as shown in SEQ ID NO: 2, or a biologically active variant thereof.

37. A method of treating or ameliorating cancer comprising:

delivering to a tumor a construct comprising a promoter which is operably linked to segment of nucleic acid which encodes a 40 kd active CPAN protein as measured on SDS-polyacrylamide gel electropheretograms and has an amino acid sequence as shown in SEQ ID NO: 2, or a biologically active variant thereof.

38. The method of claim 37 wherein the promoter is activated or derepressed in tumor cells but not in normal cells.

39. A method of treating or ameliorating the effects of ischemia comprising the step of:

delivering to cells which have been affected by ischemia a construct comprising a promoter which is operably linked to an antisense strand of a segment of nucleic acid which encodes a 40 kd active CPAN protein as measured on SDS-polyacrylamide gel electropheretograms and has an amino acid sequence as shown in SEQ ID NO: 2, or a biologically active variant thereof.

40. The method of claim 39 wherein the tumor expresses DFF45 or is transfected to express DFF45.

41. A method of identifying compounds which inhibit apoptosis, comprising the steps of:

contacting a test compound with an inactive CPAN protein and caspase-3;

testing the CPAN protein for the ability to fragment DNA, wherein a test compound which prevents caspase-3 activation of inactive CPAN to fragment DNA is a candidate compound for inhibiting apoptosis.

42. A method of making a CPAN protein, comprising the steps of:

culturing a recombinant cell in a suitable culture medium, wherein the recombinant cell comprises at least one polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1 and at least one polynucleotide comprising the nucleotide sequence of SEQ ID NO: 3, whereby a 40 kD CPAN protein and a 45 kD DFF45 protein are expressed; and

recovering CPAN protein from the cultured recombinant cell.

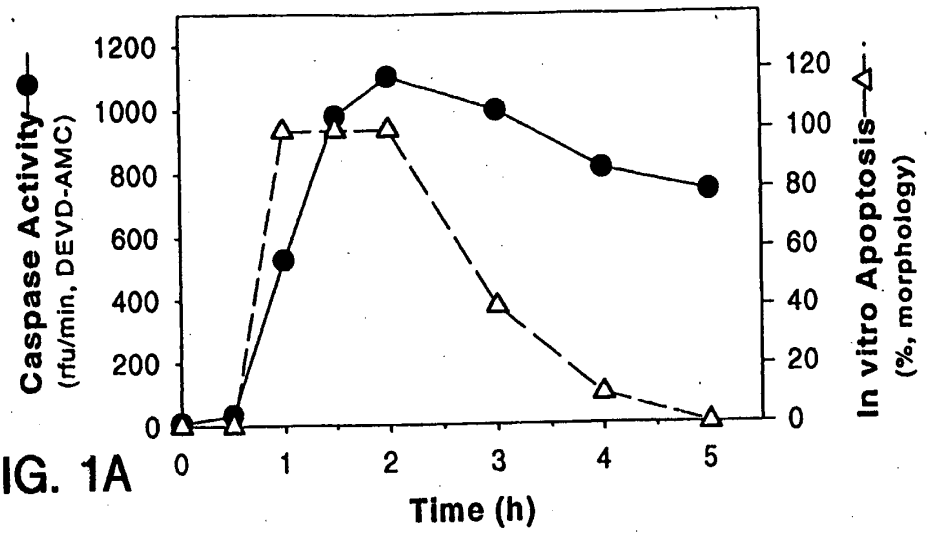

FIG. 1A

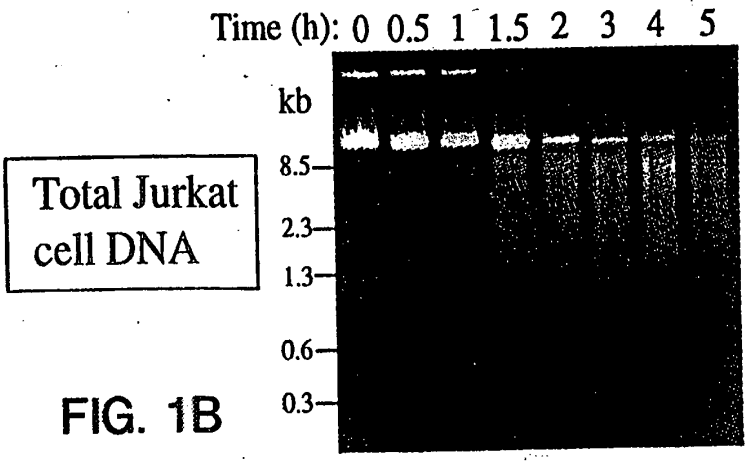

FIG. 1B

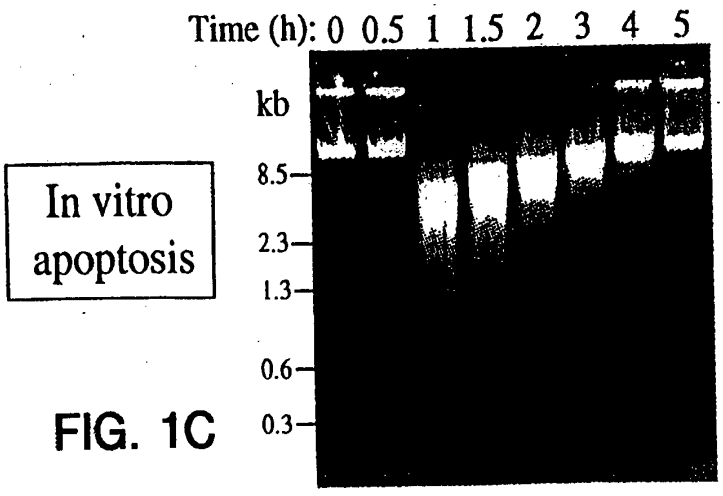

FIG. 1C

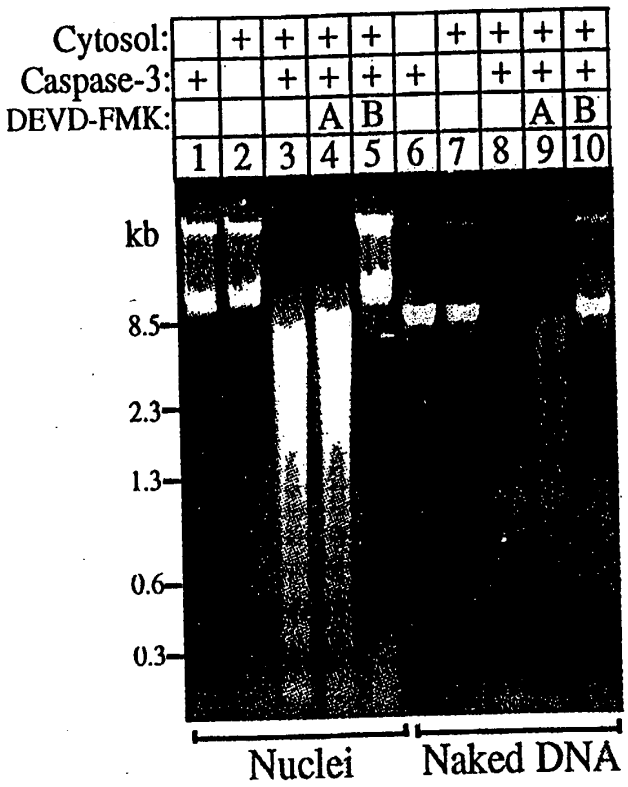
Cytosol:
Caspase-3:
DEVD-FMK:
1 2 3 4 5 6 7 8 9 10
kb
8.5
2.3
1.3
0.6
0.3
Nuclei
Naked DNA

FIG. 2

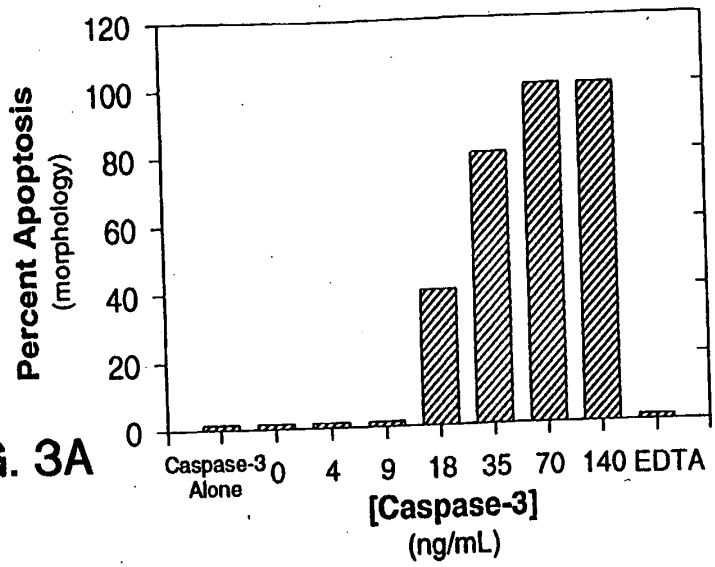
Percent Apoptosis
(morphology)
120
100
80
60
40
20
0
Caspase-3 Alone
0
4
9
18
35
70
140
EDTA
[Caspase-3]
(ng/mL)

FIG. 3A

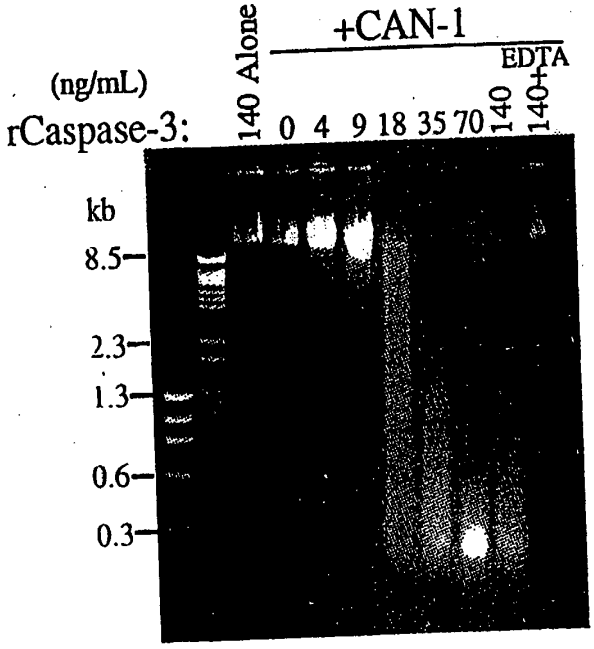
+CAN-1
140 Alone
EDTA
140+
(ng/mL)
rCaspase-3:
0 4 9 18 35 70 140
kb
8.5
2.3
1.3
0.6
0.3

FIG. 3B

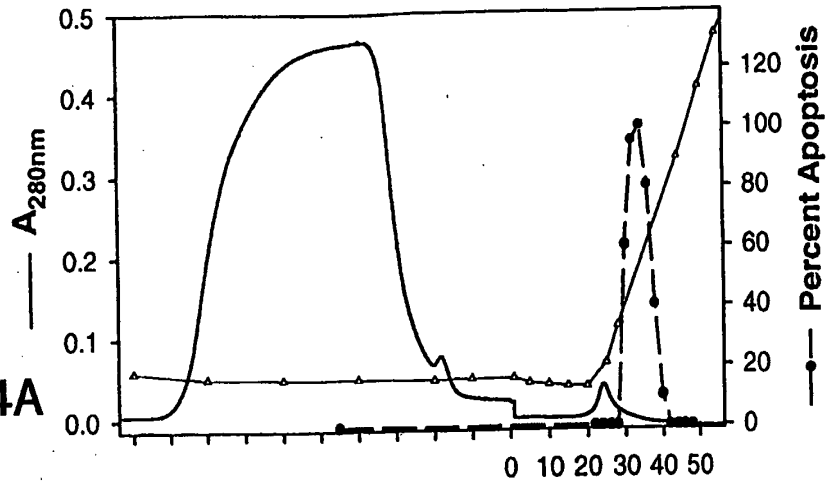

FIG. 4A

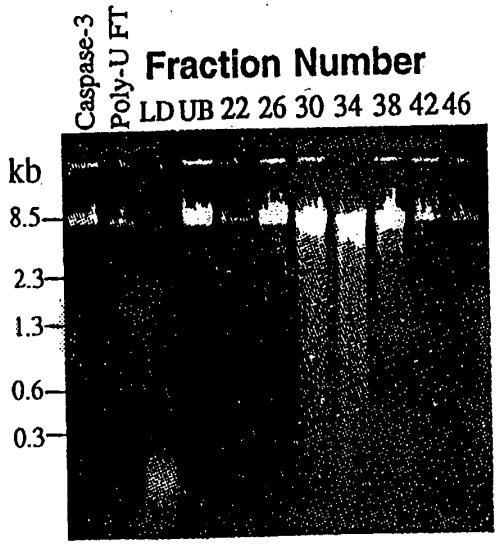

FIG. 4B

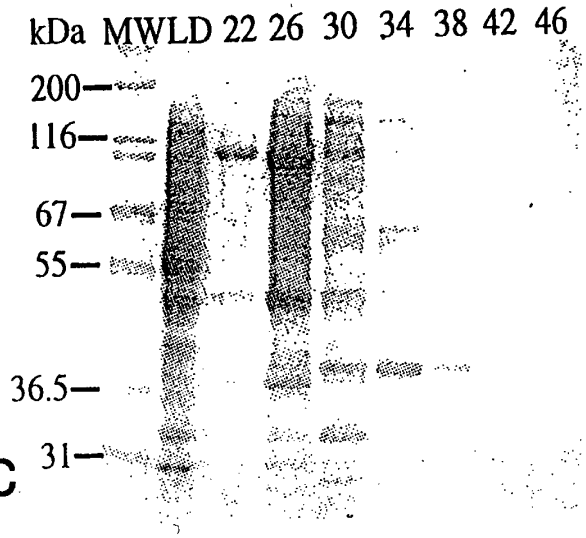

FIG. 4C

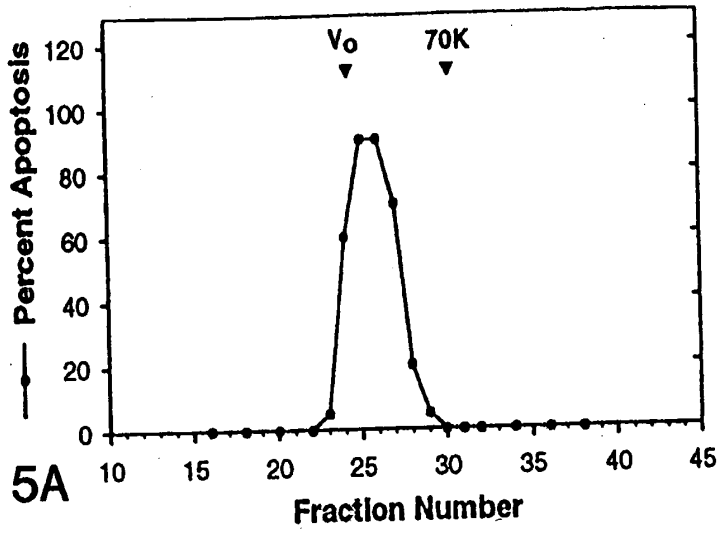

FIG. 5A

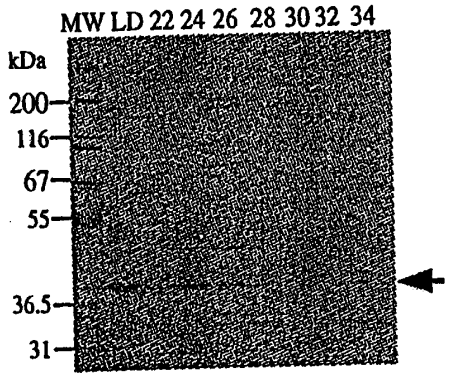

FIG. 5B

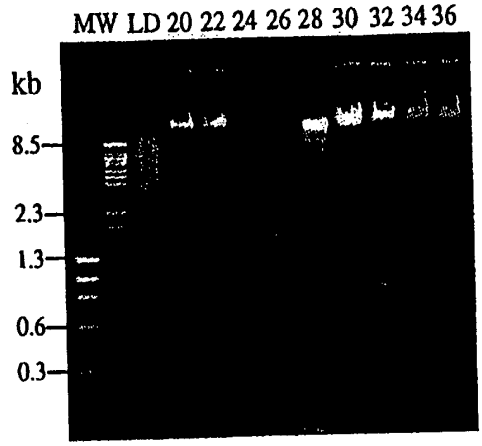

FIG. 5C

CPAN Protein Sequence

```
1   MLQKPKSVKL RALRSPRKFG VAGRSCQEVL RKGCLRFQLP ERGSRLCLYE
                I                    II
51  DGTELTEDYF PSVPDNAELV LLTLGQAWQG YVSDIRRFLS AFHEPQVGLI
                III
101 QAAQQLLCDE QAPQRQRLLA DLLHNVSQNI AAETRAEDPP WFEGLESRFQ
              IV
151 SKSGYLRYSC ESRIRSYLRE VSSYPSTVGA EAQEEFLRVL GSMCQKLRSM
                      V
201 QYNGSYFDRG AKGGSRLCTP EGWFSCQGPF DMDSCLSRHS INPYSNRESR
                                VI
251 ILFSTWNLDH IIEKKRTIIP TLVEAIKEQD GREVDWEYFY GLLFTSENLK
                VII
301 LVHIVCHKKT THKLNCDPSR IYKPQTRLKR KQPVRKRQ
```

FIG. 6

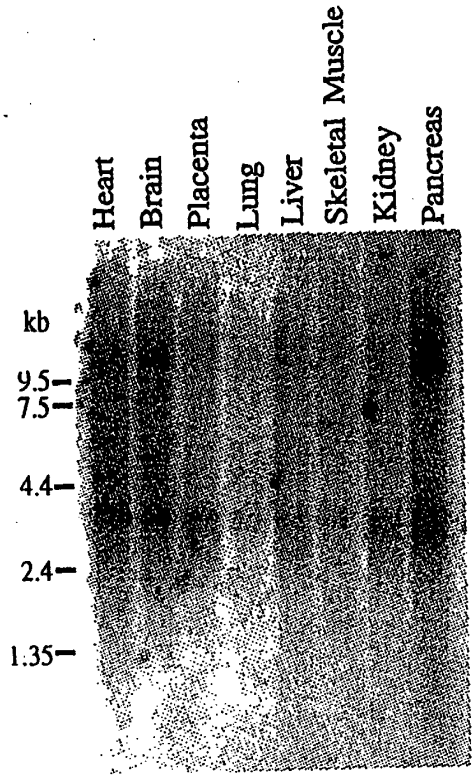
Heart
Brain
Placenta
Lung
Liver
Skeletal Muscle
Kidney
Pancreas
kb
9.5
7.5
4.4
2.4
1.35

FIG. 7A

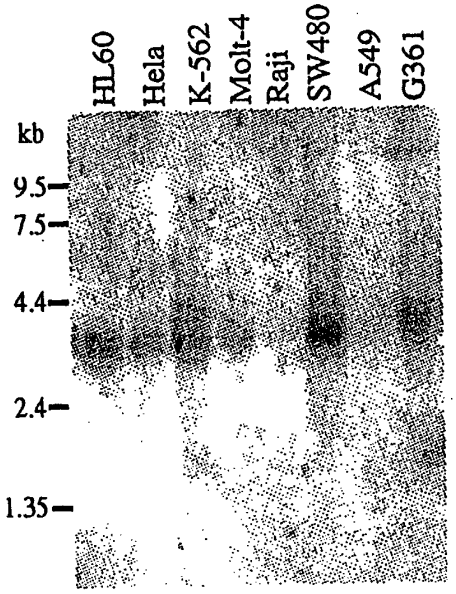
HL60
Hela
K-562
Molt-4
Raji
SW480
A549
G361
kb
9.5
7.5
4.4
2.4
1.35

FIG. 7B

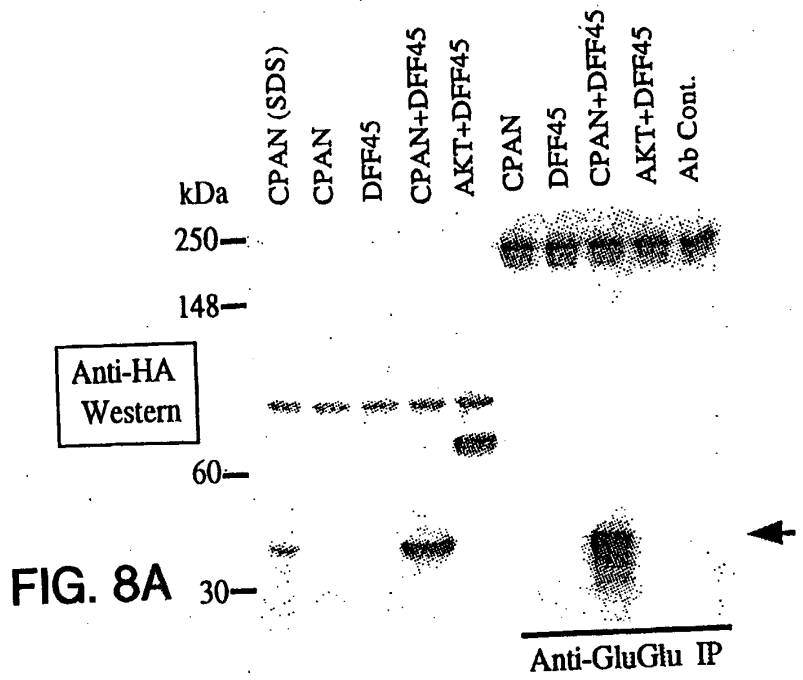
CPAN (SDS)
CPAN
DFF45
CPAN+DFF45
AKT+DFF45
CPAN
DFF45
CPAN+DFF45
AKT+DFF45
Ab Cont.
kDa
250
148
Anti-HA Western
60
30
Anti-GluGlu IP

FIG. 8A

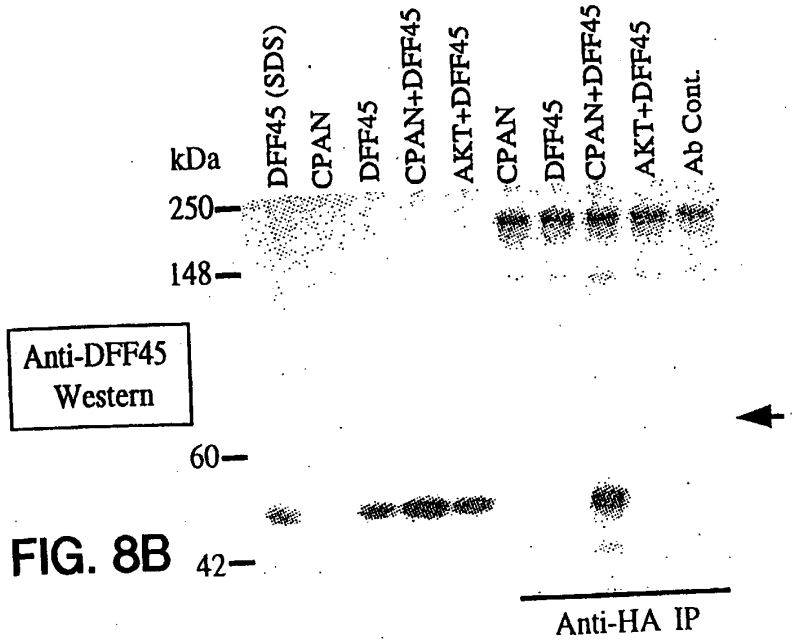
DFF45 (SDS)
CPAN
DFF45
CPAN+DFF45
AKT+DFF45
CPAN
DFF45
CPAN+DFF45
AKT+DFF45
Ab Cont.
kDa
250
148
Anti-DFF45 Western
60
42
Anti-HA IP

FIG. 8B

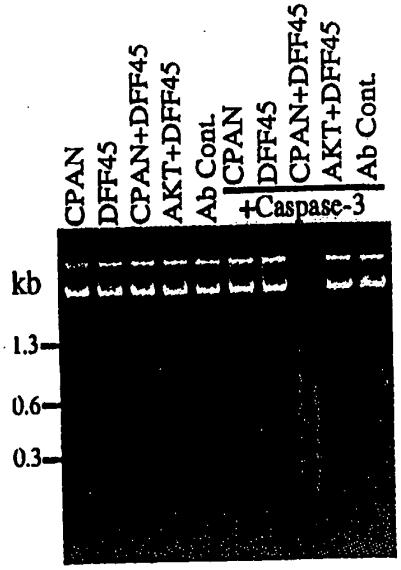
CPAN
DFF45
CPAN+DFF45
AKT+DFF45
Ab Cont.
CPAN
DFF45
CPAN+DFF45
AKT+DFF45
Ab Cont.
+Caspase-3
kb
1.3
0.6
0.3

FIG. 8C

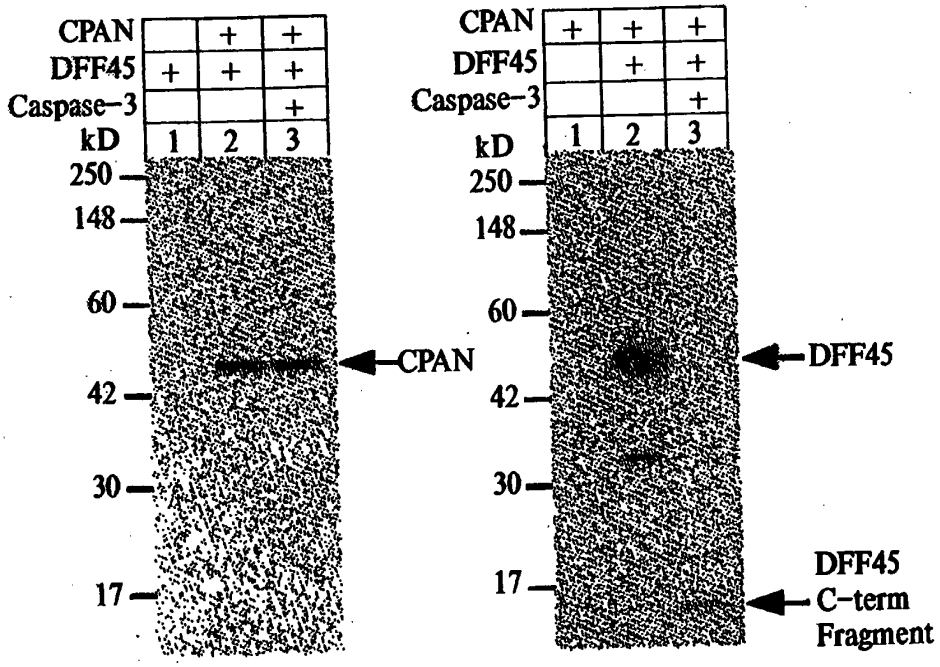
CPAN
DFF45
Caspase-3
kD
1
2
3
250
148
60
42
30
17
CPAN
CPAN
DFF45
Caspase-3
kD
1
2
3
250
148
60
42
30
17
DFF45
DFF45
C-term
Fragment

FIG. 8D

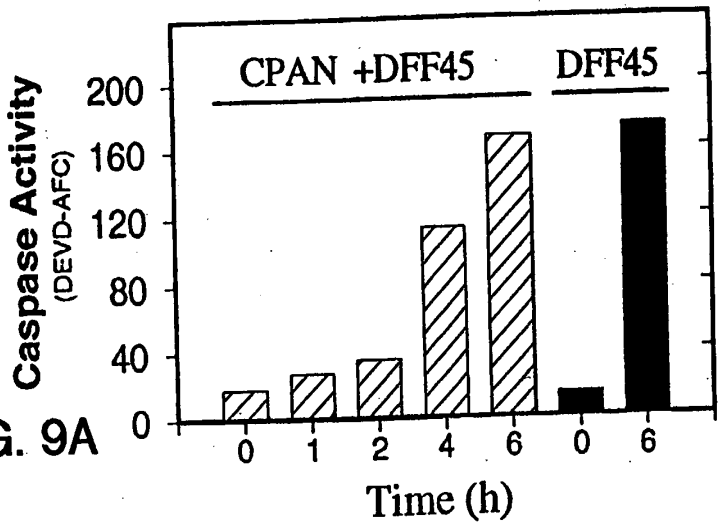
CPAN +DFF45
DFF45
Caspase Activity
(DEVD-AFC)
200
160
120
80
40
0
0 1 2 4 6 0 6
Time (h)

FIG. 9A

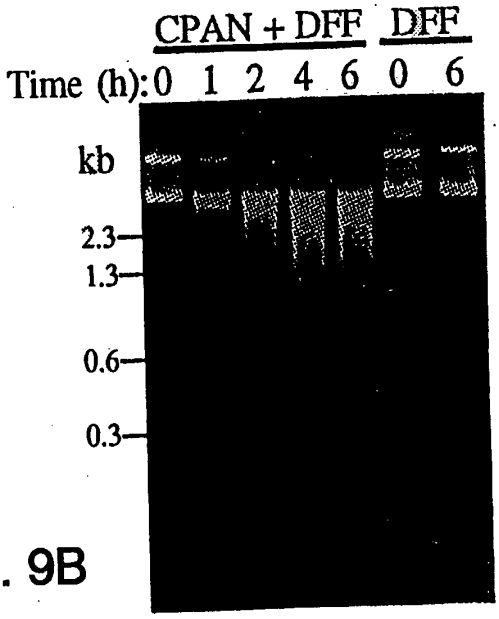
CPAN + DFF
DFF
Time (h): 0 1 2 4 6 0 6
kb
2.3
1.3
0.6
0.3

FIG. 9B

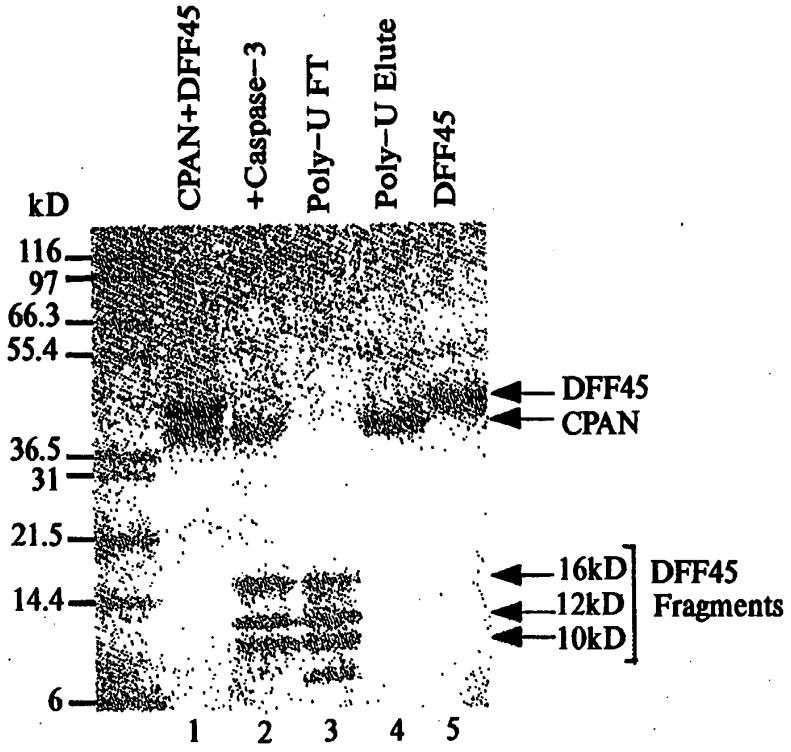

FIG. 10A

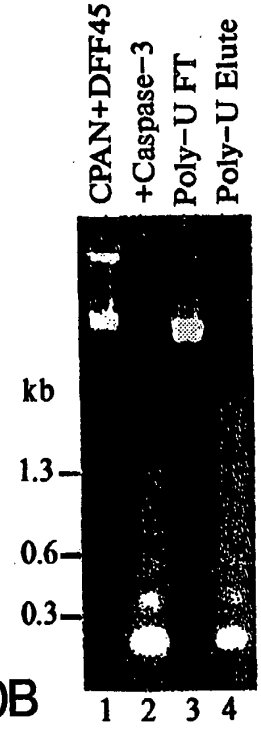

FIG. 10B

INTERNATIONAL SEARCH REPORT

Intern ial Application No
PCT/US 98/17214

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/55 C12N9/22 C12N15/12 C07K14/47 C12N5/10
C12Q1/68 C12Q1/34 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LIU X ET AL: "DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis." CELL, APR 18 1997, 89 (2) P175-84, XP002089487 UNITED STATES see the whole document	1-17, 24-36, 41,42
Y	EP 0 754 751 A (TANUMA SEIICHI) 22 January 1997 see abstract; examples 1,3	1-17, 24-36, 41,42

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 January 1999	22/01/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gurdjian, D

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern ial Application No
PCT/US 98/17214

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,P	HALENBECK R ET AL: "CPAN, a human nuclease regulated by the caspase-sensitive inhibitor DFF45." CURR BIOL, APR 13 1998, 8 (9) P537-40, XP002089486 ENGLAND see the whole document	1-14,35
P,X	MUKAE N ET AL: "Molecular cloning and characterization of human caspase-activated DNase." PROC NATL ACAD SCI U S A, AUG 4 1998, 95 (16) P9123-8, XP002089490 UNITED STATES see the whole document	1-14,35
P,X	LIU X ET AL: "The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis." PROC NATL ACAD SCI U S A, JUL 21 1998, 95 (15) P8461-6, XP002089491 UNITED STATES see the whole document	1-14,35

1

INTERNATIONAL SEARCH REPORT

Int...ational application No.
PCT/US 98/17214

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 37-40 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. al Application No
PCT/US 98/17214

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0754751 A	22-01-1997	US 5821103 A	13-10-1998
		WO 9607735 A	14-03-1996
		JP 8187079 A	23-07-1996